PHD

A study of the effect of cotreatment of taprostene (cg 4203), a novel stabilized prostacyclin analogue, with saruplase, a gene technologically produced unglycosylated single chain urokinase-type plasminogen activator (r-scuPA), in thrombolysis in vivo

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Award date:
1990

Awarding institution:
University of Bath

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for the degree of PhD

of the University of Bath

1990

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Contents

Summary

The objective of this thesis was to examine the effects of cotreatment of taprostene, a synthetic stabilised prostacyclin analogue, together with the thrombolytic agent recombinant unglycosylated single chain urokinase-type plasminogen activator (r-scuPA) in in-vivo thrombolysis.

In an open chest anaesthetized canine model r-scuPA-thrombolysis of thrombin-induced occlusive coronary artery thrombi was examined in 2 series of experiments.

- *After LAD coronary artery thrombosis, r-scuPA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$, for 60 min) restored LAD flow in 6 of 6 dogs treated, 2 of which were not analyzed after fatal ventricular fibrillation. The interval from infusion start to LAD reflow was 25 5 min ($\bar{x}$ SEM, n = 4). Compared to r-scuPA untreated animals undergoing 6 h continuous LAD ligation or stable thrombotic occlusion (n = 6) r-scuPA treated animals had a 80% smaller relative infarct size at 6 h, a more rapid rise in plasma creatine kinase (CK) activity, concurrent with reperfusion, and signs of systemic plasmin generation (partial $\alpha_2$antiplasmin and fibrinogen depletion in plasma).*

- *After 90 min of LCX coronary artery thrombosis, r-scuPA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 30 min) either alone or started together with taprostene (2 dose groups: 0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 2 h, and 0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 2 h) restored LCX flow in all 21 animals treated. Taprostene cotreatment did not significantly change the*

time required by r-scuPA to induce LCX recanalization (r-scuPA alone (group II): 22 2.1 min, n = 9 ($\bar{x}$ SEM); with low dose taprostene (group III): 18.2 1.7 min, n = 6; with high dose taprostene (group IV): 20.2 2.0 min, n = 6).

Compared with vehicle treated animals with stable LCX occlusive thrombi (group I, n = 4) and group II animals (r-scuPA alone) high dose taprostene cotreatment (group IV) exerted significant effects on the following haemodynamic parameters: ventricular contractility (decreased) and reperfusion arrhythmias (ventricular ectopic beat frequency reduced). The mean arterial blood pressure, heart rate and heart rate x pressure products were similar between all groups. High dose, but not low dose taprostene cotreatment attenuated the steep rise in CK activity on reperfusion seen in the r-scuPA alone treated group. The mean 6 h relative infarct size was smaller in group II relative to group I as a result of reperfusion induced myocardial salvage. Taprostene cotreatment further reduced enzymatic and morphological indices of infarct size. The systemic generation of plasmin by r-scuPA may have been greater in the low dose taprostene cotreatment group compared to the other 2 r-scuPA treated groups, as seen by a slight increase in the depletion of AP and plasminogen in this group.

In a rabbit model of carotid artery thrombosis the effects of infusions of r-scuPA (21.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 40 min) and a non-hypotensive dose of taprostene (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 100 min) alone, and in combination, were examined. Taprostene and r-scuPA both separately reduced the rate of occlusion and temporarily increased the flow

rate through the stenosis. Cotreatment with the 2 compounds potentiated this effect; flow was maintained at a higher level than in both separate treatment groups and no occlusion of the carotid artery occurred during the 3 h observation period. The frequency of cyclical flow variations caused by platelet aggregate formation and embolization was increased by r-scuPA but attenuated by taprostene, both alone and in combination with r-scuPA (group IV). In neither group treated with r-scuPA were fibrinogen levels affected. Taprostene reduced collagen-induced platelet aggregation in vitro (Born method). Treatment with r-scu-PA did not affect platelet aggregability here compared to vehicle controls and r-scuPA cotreatment did not alter the inhibitory potency of taprostene infusion in vivo.

Finally, the effect of taprostene infusion (80 and 400 µg/2h i.v. and placebo) on selected haemostatic and fibrinolytic parameters in healthy male volunteers was examined to determine its potential contribution to fibri- nolysis. High dose taprostene increased the endogenous fibrinolytic activity over the infusion period (statistical significance was not reached due to small number of volunteers). Plasma levels of the plasmin inhibitor AP and the clotting factor fibrinogen were unaffected. Plasma activity of the endogenous rapid plasminogen activator inhibitor (PA1-1) may have been decreased modestly by high dose taprostene infusion.
Therefore during coronary thrombolysis therapy with r-scu-PA, taprostene at non-hypotensive doses, contributes to a possible further reduction in infarct size without markedly affecting central haemodynamic parameters with the exception of arrhythmia which was reduced.

1. Introduction

1.1 Relevance of Acute Myocardial Infarction (AMI)

"Cardiovascular disease is a major cause of disability and accounts for 50 % of deaths in individuals over the age of 50 years" (Collen, Stump, Gold et al., 1988 a).

"Until comparatively recently, the epidemic of ischaemic heart disease was continuing unabated in industrialized countries, particularly among males in their peak years of economic activity. Beginning in the late 1960s, however, mortality from the disease began to recede in some countries and, in fact, has fallen by 30 % or more in Northern America and Australia. Moreover, since ischaemic heart disease is the leading cause of death in these countries, typically accounting for about one fifth of all deaths, even modest declines in mortality can result in a substantial saving of lives and an overall reduction in death rates." World health statistics annual 1987; WHO, Geneva.

1.2 Predisposing factors in thrombosis

Virchow proposed, in 1856, 3 prothrombotic changes known as Virchow's Triad:

a) changes in the vessel wall,

b) changes in the local blood flow pattern,

c) changes in the blood composition.

The experimental induction of thrombi normally requires the interaction at least 2 of these factors but Virchow's Triad simplifies aetiological analysis.

a) vessel wall changes:

Under normal conditions the vascular endothelium exhibits net non-reactivity to blood components. Prostacyclin, PGI_2 (Moncada, Gryglewski, Bunting et al. 1976; Moncada and Vane 1979), endothelial-derived relaxing factor, EDRF (Sneddon and Vane 1988) 13-hydroxy-octadecadienoic acid, 13 HODE (Buchanan, Haas, Lagarde et al. 1985) and ectoenzymes which degrade ADP and ATP (Lieberman, Lewis and Peters 1977; Bassenge and Busse 1988) have been proposed as antiplatelet metabolites of vascular origin while platelet-endothelium electrostatic repulsion has also been postulated (Sawyer 1972).

In addition to the inhibition of platelet activation and adherence, normal endothelium possesses various anticoagulant mechanisms: antithrombin activity via the release of heparan sulphate (Busch and Owen 1982), protein C activation in conjunction with thrombomodulin and protein S (Esmon 1983) and the release of plasminogen activators (Erickson, Schleef, Ny et al. 1985) (see diagram 1).

Thrombosis often occurs following mechanical and chemical damage to the endothelium, and after atherosclerotic plaque rupture which, in addition to disrupting the endothelial antithrombotic devices, expose adhesive proteins and collagen (especially types I and III), resulting in platelet adherence and aggregation. Thrombin generation is amplified by positive feedback of platelet aggregation, and with decreasing local flow rate an increasing proportion of fibrin is formed binding the platelet aggregates (Hawiger 1987, Zimmermann, Zeltsch and Lange 1979).

b) changes in local blood flow pattern:

Turbulence and stasis are associated with enhanced thrombosis and increasing shear rates promote platelet aggregation (Baumgartner, H.R. 1973). Pathologically these deviations in the normal laminar flow of blood in arteries may result following atherosclerotic and vasospastic stenosis, and occur naturally at vessel bifurcations (Born 1978).

c) changes in blood composition:

Apart from inherited thrombopathies, literature is laden with examples of acquired, induced or fluctuational changes in blood components which have been proven or inferred to render a "hyperthrombotic" condition. The early morning peak frequency in myocardial infarction onset (Muller, Rao, Greenberg et al. 1985; Muller, Tofler and Stone 1989; Willich, Linderer, Wegscheider et al. 1989; Schröder and the ISAM Study Group 1989) coincides with increased platelet aggregability (Tofler, Brezinski and Schafer 1987), reduced fibrinolytic capacity (Rosing, Brakman, Redwood et al. 1970) resulting from lower tissue plasminogen activator (tPA) levels and increased levels of its inhibitor, plasminogen activator inhibitor 1 (PAI-1) (Grimaudo, Hauert, Bachmann et al. 1988). Elevated fibrinogen, plasma factor VIIc levels and cholesterol have been shown in a prospective study to be significantly associated with an increased incidence of ischaemic heart disease (Meade, Mellows, Brozovic et al. 1986). In addition, increased fibrinogen levels cause increases in platelet aggregability (Meade et al. 1985), blood viscosity (Lowe 1985) and fibrin formation (Gurewich, Lipinski and Hyde 1976).

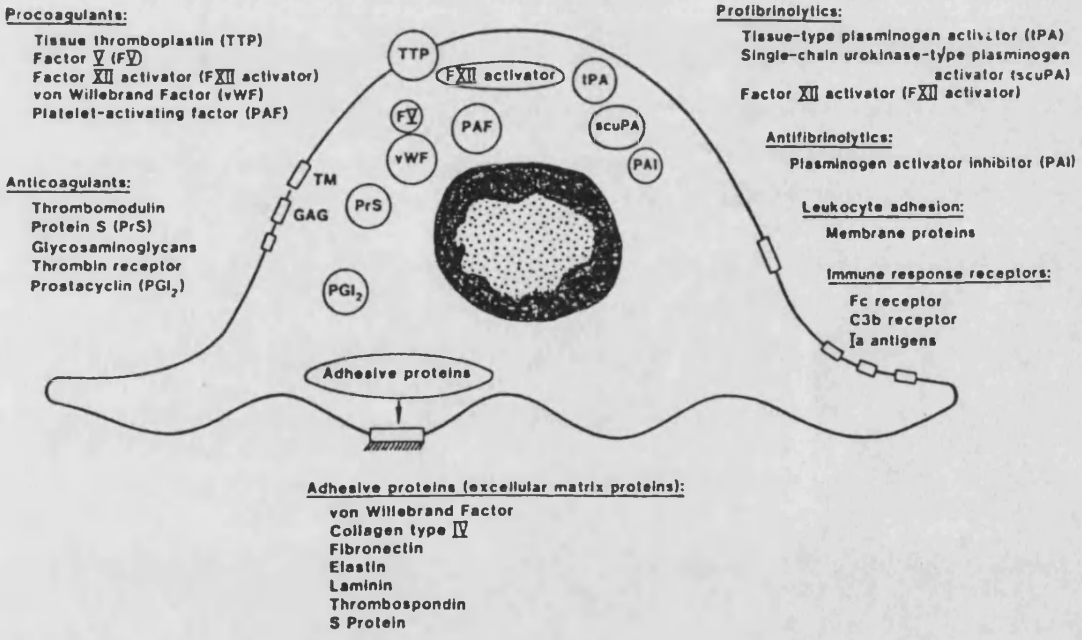


Diagram 1: Endothelial functions related to blood coagulation and fibrinolysis. Endothelial cells synthesize and release activators and inhibitors of coagulation and fibrinolysis; endothelial cells may interact with leukocytes (see section 1.6) and platelets which influence in their turn coagulation and fibrinolysis; endothelial cells present a surface for adsorption of coagulation and fibrinolysis factors entailing a balance of the coagulation and fibrinolysis systems. On the abluminal site of the cells adhesive proteins may be expressed for fixation of the cells to their substratum. (Müller-Berghaus 1987)

1.3 Acute myocardial infarction: Cause: spasm or thrombosis?

Assuming that restoring adequate blood flow to an ischaemic area of the myocardium is a valid means of limiting the loss of normal heart pump function, a knowledge of the factors predisposing, precipitating and perpetuating vessel obstruction is required before this goal, reperfusion, can be achieved efficiently. Apart from anecdotal reports most attempts to define the factors precipitating coronary artery occlusion are based on retrospective angiographical and post mortem examination. Proponents of obstruction of thrombotic origin lost resolve following the inconclusive outcome of the preliminary, poorly planned trials with streptokinase. Maseri documented vasospasm as the cause of angina at rest in all 76 patients studied, 6 of which evolved into myocardial infarction and concluded that coronary vasospasm was the precipitating (and thrombosis the perpetuating) event in this subset of patients with AMI. (Maseri, Labbate, Baroldi et al. 1978)

In contrast, De Wood et al. demonstrated arteriographically the prevalence (87%) of coronary occlusion, mostly thrombotic, within 4 hours of AMI onset and that the observed frequency of occlusions decreased to 67% over 24 hours implying spontaneous resolution. (De Wood, Spores, Notske et al. 1980). Betriu showed a 45% incidence of occlusion 1 month after infarction (Betriu, Castaner, Sanz et al. 1982).

The central idea of atherosclerotic plaque rupture and subsequent thrombosis in acute coronary artery obstruction was demonstrated by Davies and Thomas in 1985: In victims of sudden cardiac death, meticulous pathological study revealed intraluminal thrombi in 74 of 100 hearts examined. Intraintimal thrombi were seen in 21 of the remaining 26 hearts. In total, of the 115 thrombi 103 were plaque-fissure related. In this study an inherent selection criterion, mortality, may bias the results if extrapolated to include non fatal myocardial infarction.

Vasospasm may also aid plaque rupture and is therefore not exhonerated, in fact atherosclerotic arteries may tend to vasospasm as a result of deficient EDRF-mediated vasodilatory balance (Ludmer, Selwyn, Shook et al. 1986; Försterman, Mügge, Haverich et al. 1988) (see diagram 2). In this context, 2 extremely potent endogenous vasoconstrictor peptides endothelin (Yanigasawa, Kiruhara, Kimura et al. 1988) and neuropeptide Y (Franco-Cereceda, 1989) have recently been discovered. Their role(s) in ischaemic heart disease is being fervently researched at present.

The systemic elevation of specific platelet activation markers platelet factor 4 (PF4) and beta-thromboglobulin (BTG) (Kaplan and Owen 1981) in myocardial infarction (Gallino, Haeberli, Baur et al. 1985; Rasi, Ikkala and Torstila 1982; Nichols, Owen, Kaplan et al. 1982), together with the post mortem abundance of platelets in occlusive thrombi, and anterograde accumulation of platelets after occlusion (Henriksson, Edhag and Wennmalm 1985a) demonstrate a major role of platelet aggregation in coronary artery occlusion.

Fibrin binds the platelet aggregates thereby stabilizing the thrombus. Concurrent fibrinogenesis in myocardial infarction has been demonstrated by some groups by increased plasma levels of fibrin monomer and fibrin-related antigens (Kruskal, Commerford, Franks et al. 1987; Gallino, Haeberli, Baur et al. 1985; Rapold, Haeberli, Kuemmerli et al. 1989).

Current consensus of opinion, that thrombosis is the cause of the majority of prolonged coronary artery occlusions which lead to acute myocardial infarction, is substantiated by the results of recent fibrinolytic trials where fibrinolytic treatment restored coronary patency in the majority of patients, and infarction was limited (GISSI 1986, ISIS-2 1988).

The success of fibrinolytic agents in thrombolysis is attributed predominantly, if not entirely, to thrombus destabilization via fibrin solubilisation, although other actions, discussed later, may interact.

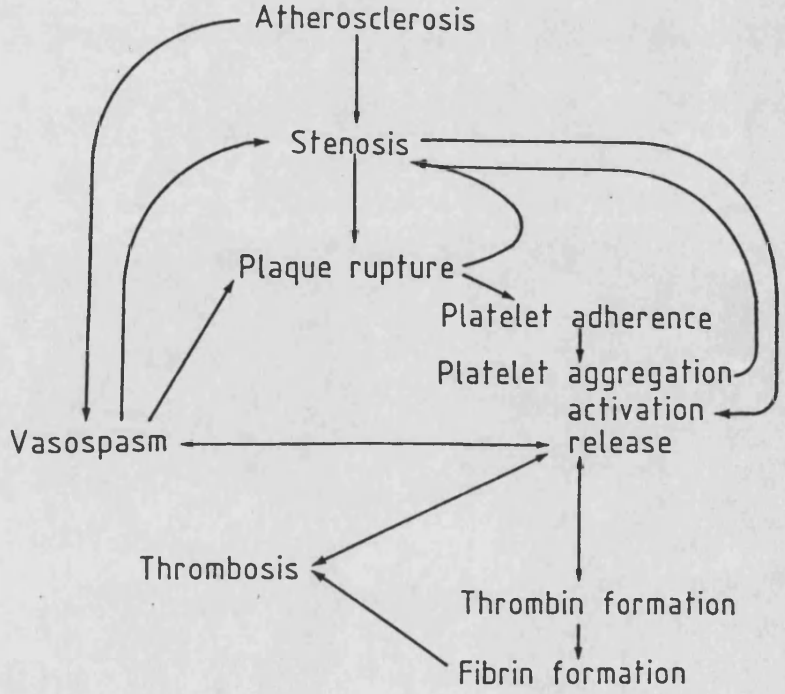


Diagram 2: Summary of proposed mechanisms of coronary artery occlusion and their interactions.

1.4 Effects of AMI

1.4.1 Functional changes

Seconds after the onset of regional ischaemia a depression of local contractile function is seen (Ross and Franklin 1976). The contraction of the ischaemic area then evolves through 4 abnormal patterns: dysynchrony, hypokinesis, akinesis and paradoxical expansion or systolic bulging (Forrester, Wyatt, Daluz et al. 1976). The net effect of regional ischaemia on ventricular function is directly related to the proportion of myocardium affected (Pfeffer, Pfeffer, Fishbein et al. 1979), at 8 % of left ventricle a reduction in diastolic distensibility is seen, above 10 % the ejection fraction is reduced, and at 15 to 17 % the left ventricular end-diastolic pressure and volume are increased. Insiduous myocardial insufficiency sets in when the abnormally contracting area exceeds 23 % of the ventricular wall, and cardiogenic shock at about 40 % (Rackley, Russell, Mantle et al. 1977). During ventricular systole the passive wall segment may exhibit compliance by bulging, which greatly exacerbates the already impaired ventricular function (Swan, Forrester and Diamond 1972) (see diagram 3). Six to eight hours later oedema and cell infiltration increase regional stiffness, which is eventually superseded by inelastic scar formation.

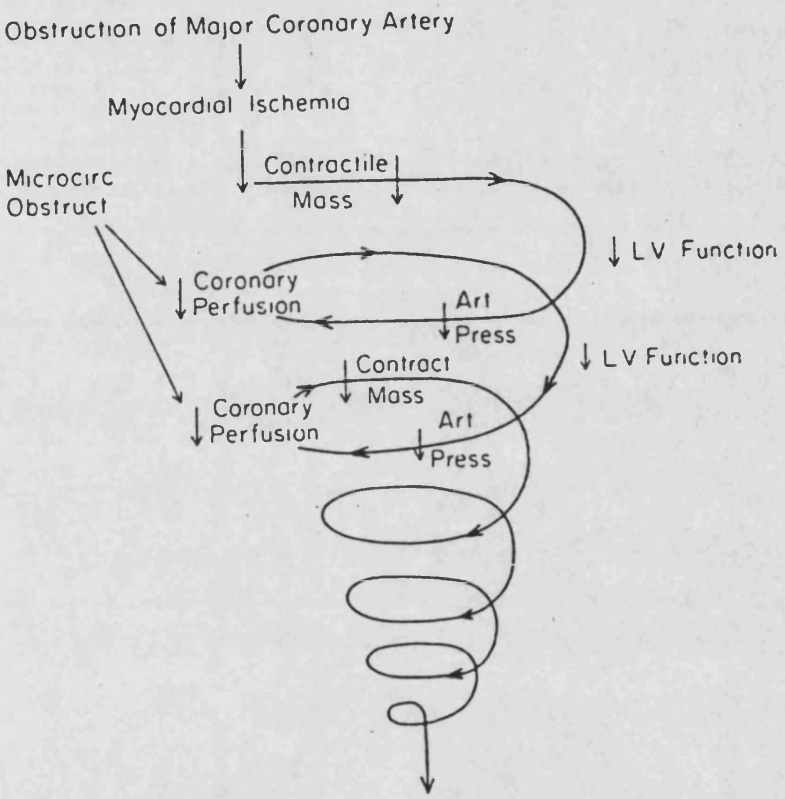


Diagram 3: Series of functional changes which may occur after coronary artery occlusion, depending on the proportion of left ventricle affected, and showing the vicious circle which may lead to cardiogenic shock.

Superimposed on a partial loss of contractile function, ischaemia-induced ventricular arrhythmias may further reduce pump efficiency. Three phases are seen after canine coronary artery ligation:

- *Initial biphasic arrhythmogenesis with peak deviant activity at 5-6 and 15-20 minutes postocclusion (Wainwright and Parratt 1988; Rosen, Janse and Myerburg 1987), often associated with ventricular fibrillation and sudden death in man (Braunwald and Sobel 1980),*
- *A second phase of arrhythmia accompanying progressing myocardial necrosis, and*
- *A later phase from 8 hours onwards with a lesser tendency to ventricular fibrillation (Campbell 1987).*

1.4.2 AMI: biochemical changes

The very high mitochondria content of left ventricular myocytes, 35 % of volume (Page and McCallister, 1973) and the precipitous fall in tissue oxygen content following ischaemia imply a high rate of oxidative metabolism closely coupled with oxygen supply. The onset of myocardial ischaemia is very rapidly accompanied by local cyanosis. The terminal components of the mitochondrial electron system become reduced under conditions of oxygen scarcity. Glycogenolysis and anaerobic glycolysis rapidly substitute the preferential fatty acid utilization and aerobic glycolysis as sources of high energy phosphates (HEP), with a resulting loss of efficiency - 2 moles of ATP formed per molecule of glucose, compared with 38 moles ATP with aerobic glycolysis. Shortly thereafter, in severe ischaemia, the anaerobic glycolytic rate slows as increasing concentrations of NADH, H^+ and lactate inhibit glyceraldehyde-3-phosphate dehydrogenase activity. Increasing tissue acidosis and citrate levels inhibit phosphofructokinase activity.

Consequently creatine phosphate levels become depleted after 3 minutes, followed by a slower fall in ATP levels (Opie 1976). Lactate accumulates rapidly as do purine bases, produced by adenine nucleotide breakdown.

The fall in contractility resulting from the imbalance in HEP supply/demand and degradation of the adenine nucleotide pool is also paralleled by an accumulation of metabolic end-products possibly to the further detriment of the contractility. These include H^+, lactate, inorganic phosphate (P_i) and products of fatty acid metabolism, as well as oxygen free radicals.

Fatty acid esters, especially lysophospholipids have been implicated in arrhythmogenesis and membrane damage due to their detergent action (Corr, Gross and Sobel 1984). Fatty acids have been shown to impair contractile function (Liedtke, Nellis and Mjos 1984), to uncouple oxidative phosphorylation (Katz and Messineo 1982) and to inhibit a number of enzymes. Ionic haemostasis is lost concurrent with HEP depletion, characterized by a cellular K^+ efflux and an increase in cytosol Ca^{2+}.

Phospholipase and neutral protease activities increase slowly leading to liberation of arachidonic acid (Chien, Han, Sen et al. 1984) and digestion of cytoskeletal proteins respectively. The latter potentially affecting plasmalemmal integrity (Steenbergen, Hill and Jennings 1985).

Accumulation of diverse catabolites causes osmotic loading of the membrane, which may be unexplainably weakened by decreased glycolysis (Ganote and van der Heide 1988).

The indirect demonstration of oxygen free radical production in the ischaemic myocardium (Rao, Cohen and Mueller 1983; Romaschin, Rebeyka, Wilson, et al. 1987) in addition to their demonstrable toxicity to liposome and erythrocyte membranes (Kellog and Fridovich 1977), microvasculature (Del Maestro, Björk and Arfors 1981), sarcoplasmic reticulum calcium transport (Hess, Okabe, Ash et al. 1984), cardiac myocytes (Burton, McCord and Ghai 1984) and on contractility (Jackson, Mickelson, Stringer et al. 1986) led to their implication in ischaemia-induced damage. Multiple sources of oxygen free radicals have been demonstrated in experimental models of ischaemia and reperfusion: via catecholamine auto-oxidation (Freeman and Crapo 1982), following conversion of hypoxanthine to xanthine, as a result of xanthine dehydrogenase conversion to

xanthine oxidase during ischaemia (Granger, Rutili and McCord 1981); by mitochondrial electron transport (Loschen, Azzi, Richter et al. 1974; Boveris 1977; Guarnieri, Muscari, Ventura et al. 1985), following cyclooxygenase action on arachidonic acid (Mason, Kalyanaraman, Tainer et al. 1980); and by NADPH oxidase in the neutrophil membrane (Babior, Rosin, McMurrich et al. 1981; Rowe, Eaton and Hess, 1984).

The higher levels of oxygen free radicals in the early stage of reperfusion, may be of greater relevance to myocardial injury and dysfunction, than those occurring during complete ischaemia.

Although the relative contribution of the various biochemical changes to irreversible ischaemic damage is still in debate (Poole-Wilson 1984; Reimer and Jennings 1986), the most certain manifestation is the loss of sarcolemmal integrity, which precedes the loss of mitochondrial function (Schaper 1987) and to which mechanical shearing may also contribute (Gamster and Kaltenbach 1979).
Following membrane disruption large intracellular molecules are released, including enzymes, creatine kinase and lactic dehydrogenase; nicotinamide adenine dinucleotide (NAD + NADH); and a mitochondrial protein which may activate complement (Rossen, Michael, Kagiyama et al. 1988; Kagiyama, Savage, Michael et al 1989). These herald the transition to the inflammatory phase.

1.4.3 ***AMI: progression and prognosis***

The two major determinants of myocyte death are the duration and severity of ischaemia (Reimer and Jennings 1979).

Duration of Ischaemia

Starting 20 to 25 minutes after coronary artery ligation in dogs a myocardial necrosis "wavefront" progresses radially, within the hypoperfused bed, until an ultimate infarct size of 30-80 % of the hypoperfused zone, or area at risk (AaR), is achieved at 6 hours (Reimer, Lowe, Rasmussen et al. 1977). Similar temporal progression of necrosis from subendocardium to subepicardium is observed in other species (Schaper 1984).

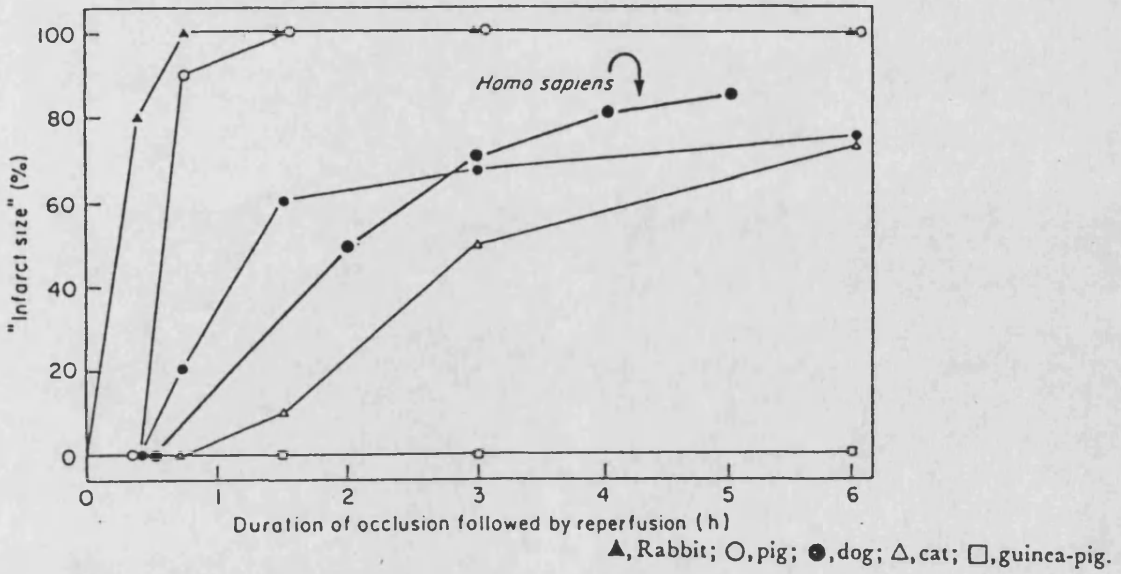


Diagram 4: The relation of infarct size (expressed as % of risk area) to the duration of ischaemia - showing species variation in the rate and ultimate extent of infarction (Hugenholtz 1988, modified from Schaper 1984).

Severity of Ischaemia

Ischaemia is defined as a subtotal or total cessation of regional perfusion, characterized not only by a severe oxygen supply/demand imbalance, as in anoxia, but also by accumulation of physio- and pathophysiological metabolites.

The rate, and ultimate extent, of transmural infarction is determined primarily by the severity of ischaemia under conditions of stable coronary artery occlusion (Maroko, Kjekhus, Sobel et al. 1971, Schaper, Binz, Sass et al. 1987). The determinants of myocardial oxygen supply and demand and hence severity of ischaemia are shown in diagram 5:

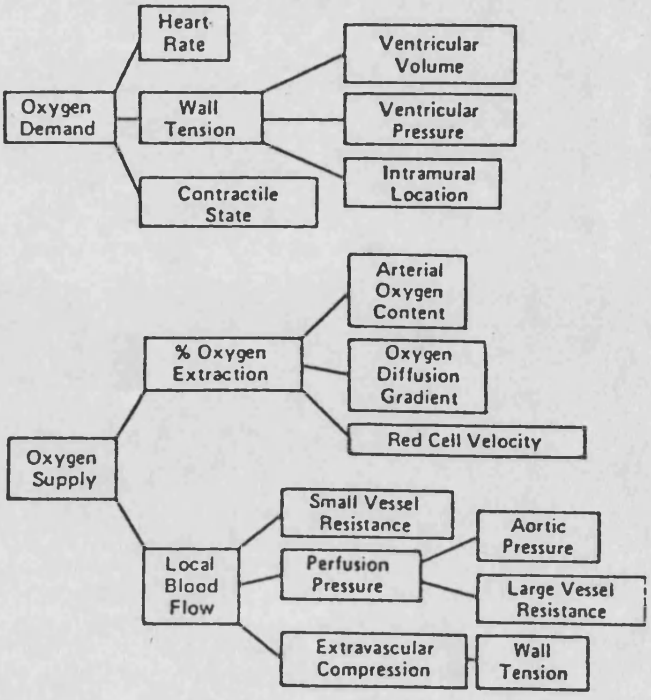


Diagram 5: Showing the major determinants of myocardial oxygen supply and demand.

The inverse relationship between wall tension and local blood flow across the myocardium explains the transmural gradient in the severity of ischaemia and the onset of myocyte necrosis in the subendocardium (Lee, Ideker and Reimer 1981). An inverse gradient relationship between HEP and lactate content of the myocardium was recorded (Murry, Reimer, Hill et al. 1985) in accordance with this observation.

Anatomical variables and infarct size

After sudden canine coronary artery occlusion, the ultimate proportion infarcted has been shown to be related to the size of the occluded artery bed or risk area (AaR) (Reimer and Jennings 1979), and the anatomical coronary artery bed involved (Becker, Schuster, Jugdutt et al. 1983; Ohzono, Koyanagi, Urabe et al. 1986). Both effects have been explained by a differing degree of residual, collateral, flow during occlusion (Reimer, Ideker and Jennings 1981; Gumm, Cooper, Thompson et al. 1988). Cohen and Rentrop, (1986) demonstrated that anginal pain, ST-ECG changes, and regional hypomotility are less prominent in patient groups with well developed collaterals following coronary occlusion. Thus the extent of existing collateral flow is a major determinant of the severity of regional ischaemia and thereby governs the rate and ultimate extent of the transmural evolution of necrosis.

Contractile state and infarct size

Under conditions of minimal collateral perfusion of an ischaemic bed secondary determinants of infarct size assume greater importance. Elevated heart rate (Przyklenk, Vivaldi, Schoen et al. 1986) and increased heart rate/blood pressure product (Schaper et al. 1987) were associated with increased infarct size. Garcia-Dorado, Theroux, Elizaga et al. (1988) showed that, for similar rate pressure product estimates of cardiac energy demand during ischaemia, hypertension caused more extensive infarction than did tachycardia.

The size of an infarct is therefore determined primarily by 4 factors arranged in descending order of importance:

- *Duration of occlusion*
- *Collateral blood supply*
- *Myocardial energy demand prior to and during occlusion*
- *Size (absolute) of the occluded bed*

Prognosis following AMI in man

In-hospital mortality, due primarily to left ventricular pump failure and arrhythmias, decreased from 30-40 % 20 years ago (Lown, Falho, Hood et al. 1976) to 10-20 % at present (Scholz, Herrmann, Tebbe et al. 1988). Attributed to this reduction are acute interventions, with the greatest success against arrhythmias: defibrillation, pacemakers and antiarrhythmic drugs. Consequently, cardiac failure, a function of infarct size now causes over 50 % of in-hospital deaths after AMI (Franzosi, Mauri, Pampallona et al. 1987). Long term mortality is affected by age, previous infarction, the site and size of infarction, the degree of mechanical dysfunction and arrhythmias at the time of discharge (Geltman, Eshani, Campbell et al. 1979; Bigger, 1986).

1.5 *Possible pharmacological interventions in AMI*

Disregarding antiarrhythmic therapy, drug intervention in AMI is aimed at reducing infarct size due to its correlation with mortality and morbidity (Caulfield, Leinbach and Gold 1976). Infarct size reduction is defined as a relative decrease in the ultimate extent of irreversible myocyte damage in treated groups compared to controls, at the end of the infarction process.

Drug interventions may be aimed at one or more of the following:

(1) Reducing energy demand

a) by reducing work by - negative inotropy

- bradycardia

- preload and afterload reduction

b) by reducing cell metabolism directly

(2) Increasing energy production potential

a) by restoring adequate artery perfusion

by - macro and micro thrombolysis

- spasmolysis

- redistribution of flow

- prevention of thrombosis and intermittant occlusions

b) by improving collateral flow contribution

by - increasing diastolic blood pressure (or)

- vasodilatation

- reduction of diastolic wall tension

- prevention of vascular compression due to oedema

c) *by increasing essential metabolite concentrations in blood*

d) *increasing glucose utilization as this is more efficient than FA oxidation*

e) *activation of pyruvate dehydrogenase*

(3) *Reducing myocardial catabolism*

by inhibition of - lipolysis
- proteolysis
- adenine nucleotide catabolism

(4) *Stabilization of cell structure and composition*

by - reduction of electrolyte migration
- membrane stabilization
- inhibition of free radical production and increased scavenging

(5) *Prevention of microvascular damage or blockage*

by - prevention of neutrophil and platelet adhesion and aggregation

(6) *Reducing the inflammatory response*

Of the many drugs which exhibit multiple antiischaemic affects in test models few are irrefutably beneficial in a clinical situation. Here the extreme variability in infarct "substrate" and timing detracts from statistical resolution, requiring very large studies. Most clinical trials have concentrated on restoring the balance of oxygen supply/ demand to the ischaemic myocardium. Calcium channel antagonist treatment, however, failed to improve any clinical endpoints (reviewed by Yusuf 1988), whereas nitrovasodilator therapy reduced the short term risk of death by 30 % (Yusuf and Collins 1985), presumably by a limitation of infarct size as measured by creatine kinase blood levels. Beta adrenoceptor antagonists, including metoprolol, propranolol and atenolol, reduced short to long term death risk, by up to 40 % in high risk subgroups, presumably by an additional antifibrillatory mechanism, by decreasing cardiac rupture (Julian, Chamberlain, Sandoe et al. 1988) and decreasing reinfarction rates (Rehnqvist and Olsson 1987). Theoretically, rapid restoration of normal coronary perfusion with accommodative flow reserve should be more rewarding than decreasing (global) energy demand or statically increasing subordinate (collateral) perfusion modes.

1.6 Coronary artery reperfusion

1.6.1 Reperfusion: theory and aim

Reperfusion, by restoring an adequate oxygen supply should halt the progression of ischaemia-induced necrosis if reversibly injured myocytes are still present at the time of institution. The resulting myocardial salvage and infarct size limitation should lead to partial alleviation of the loss of ventricular function with an attendant reduction in mortality and complications.

1.6.2 Effects of reperfusion on infarct size

Animal studies demonstrate unequivocally that reperfusion salvages jeopardized myocardium if instituted within a limited period of ischaemia. Infarcts in dog hearts reperfused at 3 hours were 10 % smaller than infarcts arising from permanent occlusion, and 60 to 70 % smaller after reperfusion at 40 minutes. No reduction in infarct size was seen by reperfusing after 6 hours (Reimer and Jennings 1979) in anesthetized dogs (Ginks, Sybers, Maroko et al. 1972; Constantini, Cordan, Lang et al. 1975) in baboons (Greary, Smith, McNamara et al. 1982) and rats (Hochmann and Choo 1987).

Effects of reperfusion on left ventricular function

The rate of contractile recovery of ischaemic segments is generally inversely proportional to the duration of the preceding ischaemia. In conscious dogs 5 and 15 minute occlusion periods required greater than 3 and 6 hours of reperfusion respectively to restore regional function even though no myocyte necrosis occurred (Heyndrickx, Millard, McRitchie et al. 1975). Lavallee, Cox, Patrick et al. (1983) demonstrated a graded rate and ultimate extent in the return of segmental contractile function following 1, 2 and 3 hours of occlusion. Bush, Buja, Samowitz et al. (1983) demonstrated that prolonged reperfusion improved contractile function after 2, but not after 4 hours of ischaemia and that this was independent of infarct size.

The delayed recovery in regional ventricular function after relatively brief periods of ischaemia, referred to as "stunning" may be attenuated in dogs by pharmacological interventions including: postischaemic coronary vasodilation (Stahl, Aversano and Becker, 1986), xanthine oxidase inhibition (Holzgrefe and Gibson, 1988), hydroxyl radical scavenging (Bolli, Wei-Xi, Hartley et al. 1987 a), iron chelation (Bolli, Patel, Wei-Xi et al. 1987 b) and iloprost infusion (Farber, Pieper, Thomas et al. 1988).

Reperfusion and myocytes

In reversibly injured myocytes reperfusion rapidly reverses ultrastructural changes (Jennings, Schaper, Hill et al. 1985), and restarts oxidative phosphorylation (Reimer, Hill and Jennings 1981) although adenine nucleotide levels may require days to recover.

Reperfusion of irreversibly injured myocytes accelerates myocyte destruction as a result of explosive swelling (Reimer and Jennings, 1985), and contraction band necrosis. K^{+} *and* Mg^{2+} *efflux, and massive* Ca^{2+} *influx, accompanied by lesser* Na^{+} *and water influx is seen. Reintroducing molecular oxygen disrupts myocyte integrity (Hearse, Humphrey and Chain 1973) in isolated hearts, and is therefore arguably coupled with the calcium influx (Hearse, Humphrey and Bullock 1987). Large intracellular enzymes, and other macromolecules are released into the circulation (Hearse, Humphrey and Chain 1973) including a mitochondrial protein which activates complement (Rossen, Michael, Kagiyama et al. 1988; Kagiyama, Savage, Michael et al. 1989).*

Polymorphonuclear leukocyte (PMN) involvement in reperfusion injury and no-reflow phenomenon

PMN depletion prior to 90 minutes ischaemia and prolonged reperfusion reduces the terminal infarct size by 30-40 % in dogs (Romson, Hook, Kunkel et al. 1983; Mitsos, Askew, Fantone et al. 1986; Jolly, Kane, Hook et al. 1983). Pretreatment with diverse antineutrophil agents may increase (Allan, Bhattacherjee, Brook et al. 1985), delay (Chambers, Yellon, Hearse et al. 1983; Klein, Pich, Bohle et al. 1988) or reduce (Bednar, Smith, Pinto, et al. 1985; Jolly and Lucchesi 1983; Mullane, Read, Salmon et al. 1984; Simpson, Todd, Fantone, et al. 1988) the evolution of necrosis, shown to be paralleled by PMN infiltration in these studies. Conversely, phorbol ester-activated PMNs reduced myocardial flow and contractile function in Langendorff-perfused rabbit hearts (Gillespie, Kojima, Kunitomo et al. 1986). In vivo, intracoronary infusion of complement C5a produced a similar contractile defect in pigs - attributed to PMN (Martin, Chenoweth, Engler et al. 1988).

Ischaemic hearts produce a chemotactic "beacon" (Hartmann, Robinson and Gunnar 1977) initially involving complement (Hill and Ward 1971) activated by heart subcellular components (Giclas, Pinckard and Olson 1979; Rossen et al. 1988), then N-formyl-methionyl-peptides of mitochondrial origin (Carp 1982), Leukotriene B_4 (Sasaki, Ueno, Katori et al. 1988) and possibly products of superoxide radical action on a plasma component (Petrone, English, Wong et al. 1980) and arachidonic acid (Perez, Weksler and Goldstein 1980).

PMN release a spectrum of inflammatory mediators including oxygen derived free radicals, arachidonic acid metabolites, platelet activating factor and lysosomal enzymes (reviewed by Lucchesi and Mullane 1986) which may contribute to the "reperfusion injury" reported by many groups.

During ischaemia, complement activation (Rossen, Swain, Michael et al. 1985; Pinckard, O'Rourke, Crawford et al. 1980) enables polymorphonuclear leucocyte (PMN) adhesion (Tonnesen, Smedley and Henson 1984) causing accumulation (Mullane, Read, Salmon et al. 1984; Engler, Schmid-Schönbein and Pavelec 1983). Reperfusion enhances PMN trapping (Engler, Dahlgren, Peterson et al. 1986 a) leading to capillary plugging (Schmid-Schönbein 1987) and associated oedema (Engler, Dahlgren, Morris et al. 1986 b). This reflow attenuation, or no-reflow phenomenon and oedema is reduced by prior PMN depletion (Engler et al. 1986 b) or inactivation (Olafsson, Forman, Puett et al. 1987).

More important from a therapeutic aspect is the recent demonstration (Litt, Jeremy, Weisman et al 1989) that reperfusion after 90 min of LCX ligation, with leucocyte filter treated blood led to a 50 % reduction in the early (2 h) infarct size (I/AaR) in dogs with low collateral flow rates. The specificity of the filters in depleting PMN is low and other (co)effects could not be excluded but the zone of no reflow was also reduced by leucopenia. Using a similar model with a 15 min period of LAD ligation Westlin and Mullane (1989) reported enhanced ventricular functional recovery and a diminished reflow attenuation in dogs with leucocyte and platelet depletion.

The relative contribution of oxygen-derived free radicals (OFR) to reperfusion injury is still in debate, as is the relative contribution of the various intra- and extracellular OFR sources. OFR scavengers may reduce reperfusion injury in blood-free Langendorff preparations (Gauduel and Duvelleroy 1984) and may even augment the myocardial salvage produced by PMN depletion in dogs (Mitsos, Askew, Fantone et al. 1986).

1.7 Fibrinolytic agents

1.7.1 General mechanism of action

The central event in fibrinolysis is the conversion of the proenzyme plasminogen to the serine protease plasmin by hydrolysis of the Arg^{560}-Val^{561} peptide bond linking the A- and B-chain. Native Glu- plasminogen is split by a variety of activators to yield two chain Glu-plasmin. The "preactivation" peptides (Glu^{75} - Lys^{76}) are subsequently cleaved by autodigestion to yield Lys-plasmin (Collen, 1980) accompanied by the unmasking of lysine binding sites, which mediate the interaction between plasminogen, fibrin and α-2-antiplasmin (AP) (Wiman, Lijnen and Collen 1979). Plasmin lyses fibrin, but lacking substrate specificity in vitro it also degrades fibrinogen and clotting factors V and VII (Collen 1980). Physiological fibrinolysis is, however, highly fibrin specific and systemic fibrinogenolysis does not occur because free plasmin is very rapidly inhibited by 1:1 complexation with AP (Mullertz and Clemmensen 1976; Moroi and Aoki 1976) (diagram 6). The plasma concentration of plasminogen (2 μM) exceeds that of AP (1 μM) and only after extensive plasmin formation do other serpins (serum proteinase inhibitors) assume relevance after complete AP exhaustion (Collen 1980). Equimolar amounts of plasminogen and AP bind, via lysine binding sites, to fibrin (Kimura and Aoki 1986) initially stabilizing the clot. With increasing plasminogen activation partial fibrin digestion causes enhanced plasminogen binding (Tran-Thang, Kruithof, Atkinson et al. 1986), which when activated overcomes local AP inhibition accelerating clot lysis (Bachman 1987).

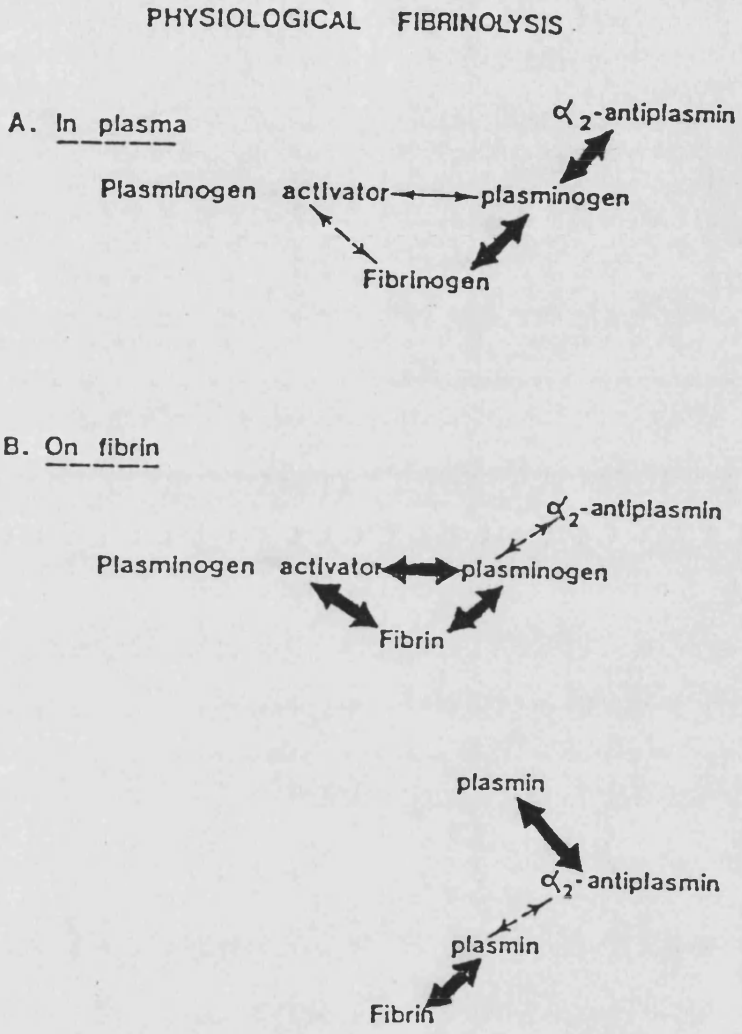


Diagram 6: Schematic representation of the interactions between fibrinogen, plasmin(ogen), alpha$_2$-antiplasmin and plasminogen activator. The size of the arrows is roughly proportional to the affinity between the different components (Lijnen 1988).

In addition, Collen (1980) calculated a half life of inhibition by AP to be 2 orders lower for fibrin bound plasmin (10 s) than for free plasmin (100 ms).

1.7.2 Streptokinase, t-PA and APSAC

Plasminogen activation may be induced therapeutically by xenobiotic molecules, streptokinase and anisoylated plasminogen-streptokinase activator complex (APSAC) or physiologically and therapeutically by tissue type and urokinase type plasminogen activators (t-PA and u-PA).

Streptokinase (SK) is a ß-haemolytic streptococci-derived protein devoid of enzyme activity. Indirect plasminogen activation results from 1:1 stoichiometic complexation of SK with human plasminogen in plasma. The complex undergoes transition to reveal a proteolytic site in the modified plasminogen moiety, and intramolecular cleavage forms a SK-plasmin complex (Reddy 1988). The plasminogen activator capacity of the former is 2 - 3 times that of the latter (Markus, Evers and Hobika 1978). Species specificity of activation is observed: Human, cat, dog and rabbit plasminogen is bound and activated by SK, whereas cow, sheep, pig, mouse and rat plasminogen does not complex with SK (Reddy 1988). Reversible p-anisoylation of the active site (Ser740) on the plasminogen-SK complex yields APSAC (anisoylated plasminogen-streptokinase activator complex) (Smith, Dupe, English et al. 1982), which still binds fibrin, but is slowly reactivated by deacylation in vivo. This prolongs the plasma clearance time and fibrinolytic activity, enabling bolus injection (reviewed by Monk and Heel 1987).

Yusuf, Collins, Peto et al. (1985) pooled the results of 24 clinical trials to confirm that intravenous fibrinolytic treatment, with diverse agents, reduced mortality by approximately 20 % after myocardial infarction. Subsequently, the two largest trials of

intravenous streptokinase showed a reduction in mortality, by about 25 % at 2 - 5 weeks (ISIS-2 1988, GISSI 1986), still evident 1 year later (GISSI 1987). Smaller studies showed a reduction in enzymatic estimate of infarct size (Serruys, Suryapranata, Simoons et al. 1987; Simoons, Serruys, Brand et al. 1986; ISAM 1986), increased ejection fraction and a trend to decreased mortality (Kennedy, Martin, Davis et al. 1987). Treatment with intravenous APSAC was reported to produce a dramatic reduction (47 %) in later mortality recorded at 1 month and even 1 year after infarction (AIMS 1988). With both agents, however, the side effects inherent to the SK moiety; antigenicity, allergic response, and rarely, anaphylaxis (ISIS-2 1988; AIMS 1988), in addition to systemic degradation of fibrinogen, plasminogen and clotting factors, has prompted the development of "pseudo-physiological" plasminogen activators with greater clot selectivity.

The two most important endogenous fibrinolytic agents are tissue-type and urokinase-type plasminogen activators (tPA and u-PA). Native tPA is a single chain serine protease released by the vascular endothelium (Rijken, Wijngaards and Welbergen 1980). Single chain tPA (sc-tPA) and plasminogen bind via kringle domains to fibrin forming a cyclic ternary complex resulting in a 400 fold increase in plasmin generation (Hoylaerts, Rijken, Lijnen et al. 1982; Ranby and Wallen 1985). sc-tPA is lysed to two-chain tPA (tc-tPA) which retains the plasminogen activator efficacy with a slight loss of fibrin specificity.

Conditioned cell culture media and recombinant DNA methods provide sufficient tPA for experimental and clinical thrombolysis.

Thrombolysis with tPA in dogs and rabbits was not associated with systemic depletion of fibrinogen, AP and plasminogen (Korninger, Matsuo, Stassen et al. 1982; Collen, Stassen and Verstraete 1983), and coronary artery recanalisation rate and frequency showed dose-dependency (Gold, Fallon, Yasuda et al. 1984; Kopia, Kopaciewicz, Fong et al. 1988). In dogs and baboons coronary thrombolysis with tPA limited the progression of myocardial necrosis (Gold et al. 1984; Flameng, v.d.Werf, Verstraete et al. 1985; Longridge, Follenfant, Maxwell et al. 1988). Despite more frequent recanalisation with tPA compared to SK (62 % versus 31 %) (TIMI 1988) only comparable reductions in 1 month mortality, 26 % for tPA (Wilcox, v.d.-Lippe, Olsson et al. 1988) were seen. The less frequent reocclusion rates associated with SK-induced systemic lytic states may contribute to this (Sherry 1988).

1.7.3 Single chain urokinase-type plasminogen activator (scu-PA)

The first reports of a urokinase-related "proenzyme" occurred in the mid seventies. Two groups (Bernick and Oller 1973; Bernick, White, Oller et al. 1974; Nolan, Hall, Barlow et al. 1977) demonstrated an enhanced ability of cell culture supernatants to activate plasminogen following their pretreatment with trypsin. Elucidation of the origin, interrelationship and identity of these variants was aided by primary structure analysis (Günzler, Steffens, Ötting et al. 1982 a and b; Steffens, Günzler, Ötting et al. 1982, reviewed in Flohé, Steffens, Günzler et al. 1985).
Native u-PA circulates in plasma at a concentration of 2 to 20 ng/ml in man (40 to 400 pM), as a high molecular weight (54 KD) single chain glycoprotein precursor, scu-PA (Bachmann 1987), known previously as pro-Urokinase (pUK or proUK) prior to the recommendation of the Subcommittee on Fibrinolysis in 1985 (Collen 1985). Cleavage at position 158 by plasmin or kallikrein produces two chain or high molecular weight urokinase (tcu-PA = HMW-UPA) (Ichinose, Fujikawa and Suyama 1986), which has 200 to 1000 times the intrinsic plasminogen activator capacity of scu-PA (Nelles, Lijnen, Collen et al. 1987; Pannell and Gurewich, 1978). The mechanism regulating the relative fibrin selectivity of scu-PA, superficially similar to that of tPA (Collen, Stassen, Blaber et al. 1984; Lijnen, De Wreede, Demarsin et al. 1984; Zamarron, Lijnen, van Hoef et al. 1984; Marder and Sherry 1988), is uncertain. scu-PA-fibrin binding affinity has been reported (Kasai, Arimura, Nishida et al. 1985; Bando, Okada and Matsuo 1987) and refuted (Stump, Thienpoint and Collen 1986).

Lijnen, Zamarron, Blaber et al. (1986) postulated that an inhibitor-scuPA complex is formed in plasma that is destabilized by fibrin. To date no inhibitior has been isolated however. Alternatively, Gurewich's group propose a mechanism which might also explain the observed time lag before scu-PA-clot lysis. This is as follows: limited plasmin digestion exposes carboxy-terminal lysine residues on fibrin which bind native Glu-Plasminogen (Harpel, Chang and Verderber 1985) inducing conformational changes which greatly enhance its susceptibility to scu-PA activation (Pannell, Black and Gurewich 1988).

Recombinant techniques involving c-DNA expression in Eschericia coli (Holmes, Pennica, Blaber et al. 1985) and hamster ovary cells (Nelles, Lijnen, Collen et al. 1987) enabled "scaled-up" production of scu-PA for characterization. These methods replace the extraction from urine (Husain, Gurewich and Lipinski 1981), plasma (Wun, Schleuning and Reich 1982 a) and kidney and endothelial cell culture media (Wun, Ossowski and Reich 1982 b). E.coli-derived scu-PA lacks the natural glycosyl group which effectively lowers the molecular weight from 54 to 47 k Daltons.

Recombinant scu-PA (rscu-PA) obtained from E.coli retains the clot selective properties of natural scu-PA (nscu-PA) but exhibits an increased catalytic efficiency (Lijnen and Collen 1988).

In a rabbit venous thrombosis model nscu-PA and rscu-PA infusion induced dose-dependent, clot-specific fibrinolysis (Gurewich, Pannell, Louie et al. 1984; Hanbücken, Schneider, Günzler et al. 1987; Collen et al. 1984). In canine (van de Werf et al. 1987; Collen, Stump, van de Werf et al. 1985) and primate (Flameng, Vanhaeke, Stump et al. 1987) coronary artery thrombosis models rscu-PA infusion ($\leq$ 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.) recanalized the artery concerned

with minimal evidence of systemic fibrinogenolysis. In the canine model under these conditions a plateau level of r-scu-PA antigen of 3.5 µg/ml plasma was observed. On stopping the infusion plasma antigen levels decreased with an initial half life of 7 minutes followed by a slower rate. In these experiments Flameng showed an attendant reduction in infarct size after r-scu-PA thrombolysis. Söhngen et al. (1988) observed a similar effect in a canine electrically-induced thrombus model, but large loading doses and high infusion rates of rscu-PA caused systemic fibrinogenolysis.

Initial clinical studies with r-scu-PA in recent myocardial infarction showed coronary recanalisation in 50 - 80 % of a total of 110 patients (van de Werf, Vanhaeke, De Geest et al. 1986 A; van de Werf, Nobuhara and Collen 1986 b; Diefenbach, Erbel, Mathey et al. 1988). α-2-antiplasmin and fibrinogen plasma levels were decreased marginally and significantly in the low and high infusion rate groups respectively, but no severe bleeding was observed.

A very recent, double blind, multicentre comparison of treatment with r-scu-PA (saruplase: 20 mg bolus, 60 mg infusion over 1 hour, both i.v.) against SK (1.5×10^6 IU i.v. infusion over 1 hour) in AMI, demonstrated multiple advantages of r-scu-PA treatment under these conditions (PRIMI Trial Study-group 1988): r-scu-PA treatment yielded a higher patency rate and earlier reperfusion (at 60 minutes: 72 % vs. 48 %, $p < 0.001$; at 90 minutes: 71 % vs. 63.9 %, $p = 0.15$). Fewer bleeding complications and a reduced clotting defect (Clauss assay) were also observed in the r-scu-PA treated patients compared with SK treated patients.

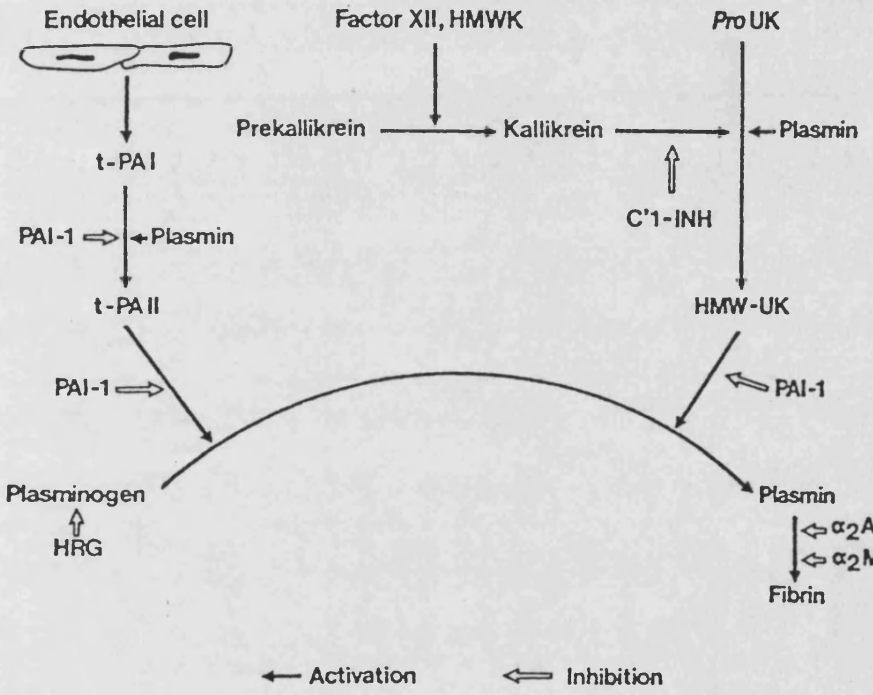


Diagram 7: The fibrinolytic system of human plasma.

t-PA 1 : single-chain tissue-type plasminogen activator (sct-PA)
t-PA 2 : two-chain t-PA (tct-PA)
PAI-1 : plasminogen activator inhibitor
HMWK : high molecular weight kininogen
Pro UK : pro-urokinase, single chain urokinase-type PA (scu-PA)
HMW-UK : high molecular weight urokinase, two chain u-PA (tcu-PA)
C'1-INH: C1-inhibitor
α_2AP : alpha$_2$-antiplasmin
α_2-M : alpha$_2$-macroglobulin
HRG : histidine-rich glycoprotein
(Bachmann 1987)

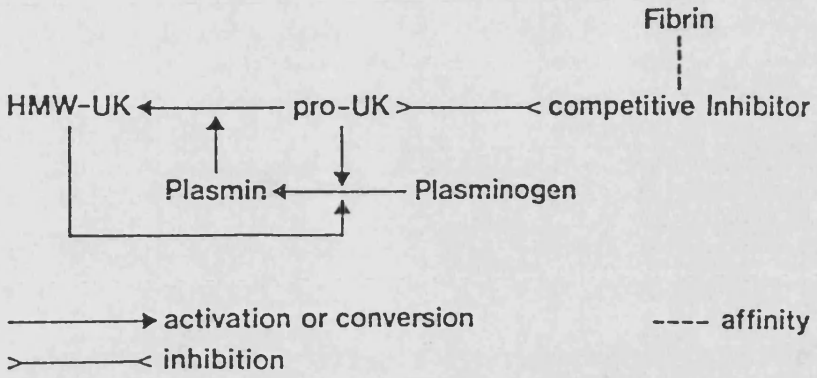


Diagram 8: Hypothetical model to explain the fibrin specificity of scu-PA (proUK) (Lijnen and Collen, D. 1986).

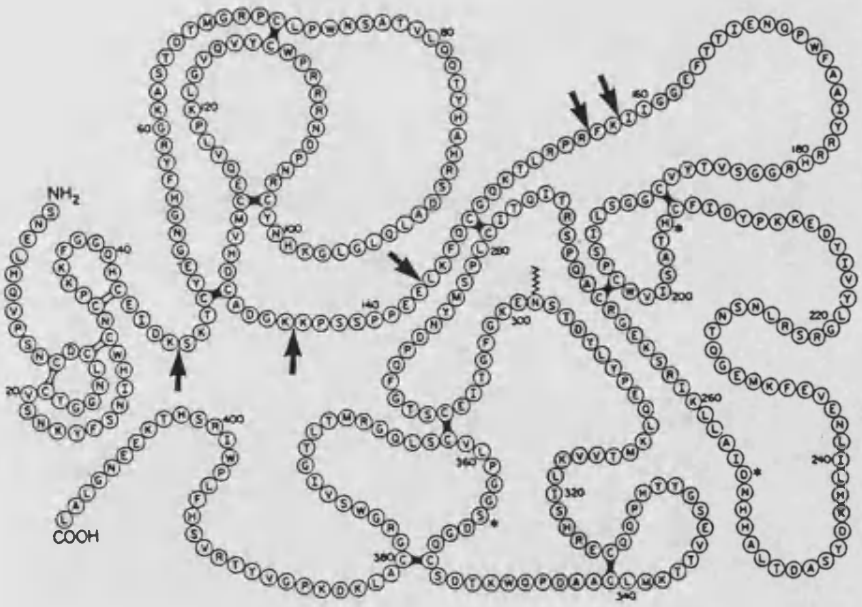
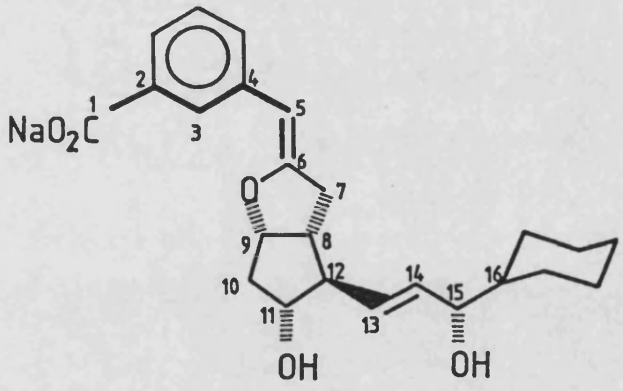


Diagram 9a: Two-dimensional structure of human single-chain urokinase-type plasminogen activator (scu-PA). The arrows indicate cleavage sites for various proteolytic enzymes (see text). Cleavage of the Lys_{158}-Ile_{159} bond results in the active two chain form. The zigzag line indicates the N-glycosylation site, and the stars show the three active site amino acid residues. (Modified from Holmes et al. 1985)

1.8 Prostacyclin and analogues (including taprostene)

The discovery of prostacyclin (PGI_2) (Moncada et al. 1976) and recognition of its potent vasodilator, antiplatelet and cytoprotective effects (reviewed by Vane, J.R. 1985) precipitated a search for analogues with enhanced chemical and metabolic stability. This yielded a number of prostacyclin mimetics including iloprost (ZK 36374) and taprostene (CG 4203).

Compared with PGI_2, taprostene exhibits a relative platelet anti-aggregatory potency of 0.46 (in vitro), an hypotensive potency of 0.14 (anaesthized rat i.v.) and a four-fold longer duration of antiplatelet action after bolus injection i.v. (Flohé, Böhlke, Frankus et al. 1983) (reviewed Lefer and Darius 1989).



CG 4203
TAPROSTENE

Diagram 9b: Structure of taprostene

1.8.1 Effects of PGI_2 and analogues on AMI

3 lines of evidence point to prostacyclin as being an important antiischaemic mediator.

(1)

Atherosclerosis, the major pathological substratum in AMI, may lower vascular PGI_2 production in animals (Dembinska-Kiec, Gryglewski, Zamuda et al. 1977) and in man (Sinzinger, Feigl and Silberbauer 1979).

(2)

During myocardial infarction in man (Henriksson, Wennmalm, Edhag, et al. 1986; Fitzgerald, Roy, Catella et al. 1986) and animals (Karmazyn 1986; Prosdocimi, Finesso, Gorio et al. 1985) local prostacyclin production is increased. In platelet free systems, cyclooxygenase inhibition may worsen the contractile defect resulting from ischaemia, and exogenous PGI_2 replacement is palliative (Schrör 1987; Berti, Rossoni, Magni et al. 1988).

(3)

In experimental myocardial ischaemia exogenous prostacyclin and mimetics show beneficial effects on various infarction indices including: electrocardiographic (Muller et al. 1984; De Langen, Van Gilst and Wesseling 1985; Ogletree, Lefer, Smith et al. 1979), enzymatic (Müller et al. 1984; Araki and Lefer 1980; Schrör, Darius, Ohlendorf et al. 1982; Ogletree et al. 1979), contractile (Araki and Lefer 1980; Nayler, Purchase and Dusting 1984; Farber et al.

1988) and morphological (Jugdutt, Hutchins, Bulkley et al. 1981; Melin and Becker 1983; Simpson, Mitsos, Ventura et al. 1987). Furthermore during ischaemia an antiarrhythmic and antifibrillatory action of PGI_2 and mimetics has been reported for non-hypotensive doses in rats (Müller et al. 1984; Johnston, Macleod and Walker 1983), dogs (Au, Collins, Harvie et al. 1979; Starnes, Primm, Woosley et al. 1982; Fiedler and Mardin 1986) and pigs (De Langen et al. 1985).

Numerous diverse cardioprotective mechanisms of PGI_2 and mimetics in ischaemia and reperfusion have been proposed:

(1)

Preload and afterload reduction and hence reduced myocardial oxygen demand (Lefer, Ogletree, Smith et al. 1978).

(2)

Increased regional blood supply via collateral vasodilation (Judgutt et al. 1981; Lupinetti, Starnes, Laws et al 1986).

(3)

Inhibition of platelet activation and trapping in the ischaemic myocardium (Ogletree et al. 1979; Michael, Hunt, Lewis et al. 1986).

(4)

Inhibition of ischaemia-enhanced catecholamine release within the myocardium (Schrör, Addicks, Darius et al. 1981; Funke and Schrör 1984; Schrör and Funke 1985).

(5)

Inhibition of intracellular lysosomal membrane disruption and release of potentially deleterious enzymes (Ogletree et al. 1979, Hieda, Toki, Sugiyama et al. 1988).

(6)

Preservation of myocyte superoxide free-radical scavenging potential (Thiemermann, Steinhagen-Thiessen and Schrör 1984).

(7)

Preservation of high energy phosphate reserves (Gercken, Gallenkämper, Schrör et al. 1982; Pissarek, Goos, Nöhring et al. 1987).

(8)

Cytoprotection or reduction of myocyte membrane degradation and disruption by undefined mechanism(s) (Araki and Lefer 1980; Smith, Kloster, Stocklin et al. 1984; Müller, Schneider, Hennies et al. 1984; Darius, Osborne, Reibel et al. 1987). The effect is not confined the myocardium as taprostene exhibited antiproteolytic effects in pancreas homogenates (Bitterman, Smith, Lefer et al. 1988).

(9)

Inhibition of neutrophil mediated injury (Simpson et al. 1987). Prostacyclin inhibits PMN adhesion to endothelial cells (Boxer, Allen, Schmidt et al. 1980; Zimmerman, Wiseman and Hill 1985 a) and has been shown by some (Simpson et al. 1987; Smolen, Korchak and Weissman 1980; Fantone and Kinnes 1983), but not all (Boxer et al. 1980; Gryglewski, Szczeklik and Wandzilak 1987) to attenuate superoxide release. Taprostene (Stahlberg, Loschen and Flohé 1988) showed potent inhibition of formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated cytochrome-C reduction by human PMN in vitro ($IC_{50} \triangleq 5 \times 10^{-7}$ M). PMN migration is inhibited by PGI_2 in vitro (Zimmerman et al. 1985 a) and in vivo (Simpson et al. 1987; Rampart and Williams 1986).

1.9 Rationale for coadministration of taprostene with scu-PA

Taprostene, and other PGI_2 mimetics, may exert many pharmacological effects of potential value in thrombolysis. Schumacher (1985) showed that PGI_2 and/or heparin cotreatment with SK increased the reflow rate in a canine coronary thrombolysis model, and PGI_2 diminished the intermittent reocclusions seen with SK alone. PGI_2 did not shorten the time to recanalisation. Infarct size was unfortunately not recorded. Prostaglandin E_1 (PGE_1) enhanced the urokinase lysis of fresh, but not matured, platelet-fibrin thrombi in vitro (Terres, Beythien, Kupper et al. 1988; Terres, Beythien, Kupper et al. 1989), and after PGE_1 coinfusion with tPA in rabbits accelerated fibrin degradation was recorded (Vaughan, Plavin, Schafer et al. 1988). Sharma, Wyeth, Heinemann et al. (1988) demonstrated enhanced ventricular function and short term coronary patency after intracoronary PGE_1 together with SK in AMI patients.

The effects of fibrinolytic agents on platelet activity are complex. Fibrin degradation products (FDP) (Kopec and Latallo 1978) and plasmin mediated degradation of platelet glycoprotein receptors (GP), fibrinogen, adhesive proteins and clotting factors may impair platelet function and clotting (Marder and Sherry 1988). Conversely reports of platelet hyperaggregability after SK and tPA infusion in rabbits (Ohlstein, Storer, Fujita et al. 1987) and in man (Fitzgerald, Catella, Roy et al. 1988), and ex vivo treatment of platelets (Fitzgerald and Fitzgerald 1987), may be a result of prothrombin activation (Seitz, Blanke, Praetorius et al. 1988) or increased thromboxane levels (Fitzgerald, Catella, Roy et al. 1988).

Thrombin inactivates scu-PA (Gurewich and Pannell 1987). Heparin therapy which itself augments scu-PA activation (Lijnen and Collen 1986), may also promote platelet aggregation (Salzman, Rosenberg, Smith et al. 1980; Barradas, Mikhailidis, Epemolu et al. 1987; Cola and Ansell 1990). Prostacyclin's anti-platelet effect is complemented by its potentiation of heparin's anticoagulative effect (Miletich, Jackson and Majerus 1978). By inhibiting platelet release, taprostene may influence the dynamic balance of thrombosis and thrombolysis: α-granules contain both a) fast acting plasminogen activator inhibitor (PAI-I) (Kruithof, Nicoloso and Bachmann 1987), which inhibits two-chain urokinase (tcu-PA) (Bachmann 1987) and endogenous t-PA, and b) platelet factor 4, which exerts modest antiheparin effects (Niewiarowski 1977). Severe stenoses, as in incomplete thrombolysis, cause the attachment and progressive growth of platelet aggregates (Badimon, Badimon, Turitto et al. 1987; Uchida, Yoshimoto and Murao 1975; Folts, Crowell, Rowe, 1976). The platelet thrombus may break off and regrow repeatedly producing a "saw toothed" flow pattern, culminating in permanent vessel occlusion (Falk 1985). PGI_2 not only prevents the experimental formation of coronary thrombi (Romson, Haack, Abrams et al. 1981; van der Giessen, Mooi, Rutteman et al. 1984) but also abolishes the cyclical flow phenomenon (Aiken and Shebuski 1980). Although platelet depletion in dogs does not affect infarct size after non-thrombotic occlusion and reperfusion (Mullane and Mc Giff 1985), and ibuprofen salvages myocardium independent of antiplatelet action (Romson, Hook, Rigot et al. 1982), hypercholesterolemia in rabbits causes a platelet-associated increase in both infarct size and no-reflow phenomenon (Golino, Maroko and Carew 1987).

Therefore, while antiplatelet agents probably do not have a marked and direct effect on infarction produced by mechanical occlusion of the artery, they may prolong the salvaging effect of fibrinolytic agents by maintaining patency, ideally until supportive regional collateral supply develops. This is exemplified in the ISIS-II study in which aspirin cotreatment further augmented the SK reduction in mortality from 23 % (SK alone) to 39 % (SK + aspirin) and the excess reinfarction rate in the SK group was reduced to a level below that recorded in the aspirin alone group. The demonstration of EDRF's antiplatelet properties (Radomski, Palmer and Moncada 1987 a, b), its synergy with PGI_2 here (MacDonald et al. 1988) and the free radical inactivation of EDRF (Moncada, Palmer and Higgs 1987) is interesting insofar as PGI_2 analogues including taprostene may limit OFR production by PMN (Stahlberg, Loschen and Flohé 1988).

Prostacyclin coadministration may have another aspect: In patients with peripheral arteriovascular disease prostacyclin infusion was reported to increase endogenous fibrinolytic activity, as estimated by the euglobulin clot lysis time (ECLT) (Dembinska-Kiec, Kostka-Trabka and Gryglewski 1982; Szczeklik, Kopec, Sladek et al. 1983; Musial, Wilczynska, Sladek et al. 1986). Winther, Snorrason, Knudsen et al. (1987) suggested an enhanced tPA production mechanism and Levin and Santell (1988) showed that elevated cAMP may promote, or reduce, the expression of tPA after stimulation of endothelial cells in vitro. Prostacyclin (Hussaini and Moore 1985) and taprostene (Schneider 1987) infusion in rats has been shown to increase

endogenous fibrinolysis dose-dependently, although in perfused rat hindlimbs peptidoleukotriene - but not PGI_2-infusion, liberated tPA (Tranquille and Emeis 1988). As tPA has been shown to act synergistically with scu-PA in vivo (Collen, Stassen, Stump et al. 1986; Collen, Stassen and De Cock, 1987; Gurewich and Pannell 1986; Ziskind, Gold, Yasuda et al. 1989) this effect may be of significance in prostacyclin analogue-scu-PA cotreatment.

1.9.1 Potential limitations and side effects of combination therapy in AMI

During myocardial infarction platelet sensitivity to inhibition by PGI_2 may decrease (Müller, Rao, Greenberg et al. 1985; Jaschonek, Karsch, Weisenberger et al. 1986), as it does in unstable angina (Swan and Wallentin 1987) and after tPA infusion in rabbits (Shebuski and Ohlstein 1987). Prolonged PGI_2 infusion (> 12 hours) is often accompanied by waning efficacy (Sinzinger, Silberbauer, Horsch et al. 1981) and even rebound hyperaggregability after cessation of infusion (Dembinska-Kiec, Zmuda, Grodzinska et al. 1981).

Common to all vasodilators is the potential to produce coronary flow maldistribution ("steal"). This has been reported by one group after infusion of iloprost in A.M.I. patients (Bugiardini, Galvani, Ferrini et al. 1987) but not mentioned by others infusing iloprost or PGI_2 (Henriksson, Edhag, Edlund et al. 1985 a; Bergman, Daly, Atkinson et al. 1981; Firth, Winniford, Campbell et al. 1983; Swedberg, Held, Wadenvik et al. 1987).

1.10 Aim

The aim of this research is to answer the following questions experimentally:

(1)

Does taprostene cotreatment affect the thrombolytic rate, extent or frequency of recanalisation seen with scu-PA alone?

(2)

If so, is this accompanied by deleterious, or beneficial, effects on haemostatic and haemodynamic parameters?

(3)

Is the established myocardial protective effect of prostacyclin and mimetics during ischaemia and reperfusion still observed when: the infusion is started late in the ischaemia phase but before reperfusion, in an animal model of thrombotic occlusion undergoing fibrinolytic therapy?

(4)

Does taprostene contribute to the maintenance of arterial patency above and beyond that of fibrinolytic therapy?

(5)

What is the effect of taprostene alone on the endogenous fibrinolytic system in man and is this relevant to clinical fibrinolysis?

2.0 Materials and methods

2.1 Materials

2.1.1 Drugs tested

Recombinant single-chain urokinase type plasminogen activator of recombinant origin (r-scu-PA) was obtained from Eschericia coli by expression of c-DNA coding for full length unglycosylated scu-PA (411 amino acid chain, 46,420 Daltons). A specific activity of 161,000 IU/mg protein was recorded after plasmin activation and subsequent incubation with the chromogenic peptide substrate (S-2444, Kabi Vitrum) (Dr. W. Günzler, Grünenthal, personal communication 1986) (see diagram 9a).

Taprostene - CG 4203 - [5Z, 9α, 11α, 13E, 15S)-2, 3, 4-Trinor-1,5-inter-(m-phenylene)-6,9 epoxy-11,15-dihydroxy-15-cyclohexyl-16, 17, 18, 19, 20-penta-nor]-prosta-5,13-dien-1-oic acid, monosodium salt (Flohé et al. 1983) was dissolved in 1.3 % sodium bicarbonate solution prior to infusion (see diagram 9b).

2.1.2 Chemicals and Reagents

Unless otherwise specified all chemicals and reagents were obtained from Merck, Darmstadt, FRG.

Suppliers of the exceptions were:

- *Boehringer Mannheim, FRG: Citrate buffer (0.11 M), Owren's veronal buffer, human thrombin (fibrinogen a reagent), Platelet factor 4 (PF4) ELISA kit, creatine kinase assay kit (CK-NAC)*
- *Kabi Vitrum, Munich, FRG: chromogenic substrates S-2444 and S-2251, human plasmin*
- *Cheva, Bad Segeberg, FRG: pentobarbitone (Nembutal)*
- *Bio Mérieux, Asnières, France: bovine thrombin*
- *Fluka, Switzerland: heparin, sodium salt*
- *Serva, Heidelberg, FRG: ε aminocaproic acid*
- *Diagnostica Stago, France: CTAD tubes*

Reference compounds and standards:

- *fibrinogen standard and platelet factor 4 assay kit: Boehringer Mannheim, FRG*
- *PAI-depleted plasma: Kabi Vitrum, Munich, FRG*
- *Collagen: Hormon Chemie, Munich, FRG*

2.1.3 Equipment

Haemodynamic measurements and ECG recordings were carried out using equipment described in the Methods section.

- *Photometer: Model 1101, Eppendorf FRG, maintained at 37°C 1°C with a heated air hood for ECLT assay*
- *Coagulometer: Amelung FRG, using plastic tubes, 3.5 ml from Sarstedt, FRG*
- *Blood gas analyser: Model ABL2, Radiometer, Copenhagen, Denmark*
- *Infusion pumps: Perfusor VI, Braun, Melsungen, FRG*
- *Planimeter: Kontron, Munich, FRG*
- *Respirator: developed within Grünenthal, Aachen, FRG*
- *Blood pressure transducers and tip manometer were calibrated weekly with an open ended mercury manometer*

2.2 Methods

2.2.1 Dog coronary artery thrombosis models

3 experimental fibrinolysis protocols were employed in 2 canine open-chested coronary artery thrombosis models (Diagram 10):

2.2.1.1

Left anterior descending coronary artery (LAD) model:

Aim:

to establish a coronary artery thrombosis model, then to determine:

- *the stability of untreated thrombi produced by the method used over 6 hours*
- *the effect of r-scu-PA treatment (alone) in this model and hence to fix an experimental platform upon which r-scu-PA and taprostene cotherapy could be tested in later experiments (diagram 10)*

3 animal groups were compared:

a) *LAD ligation for 6 hours (group a)*

b) *LAD thrombosis: untreated controls (group b)*

c) *LAD thrombosis: treated with intravenous r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 60 minute duration) started 90 minutes after onset of ischaemia.*

2.2.1.2

Further LAD thrombosis studies with a prolonged ischaemic phase together - prior to thrombolytic treatment - with antiarrhythmic prophylaxis (diagram 10);

Aim:

to decrease the proportion of animals failing to complete the experiment as a result of ventricular fibrillation

r-scu-PA infusion was started 120 minutes after LAD thrombotic occlusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 30 min duration)

and

lignocaine was administered to attenuate ventricular fibrillaton (75 mg loading dose at the onset of ischaemia, and 3 $mg \cdot kg^{-1} \cdot min^{-1}$ i.v. infusion for 120 minutes started with r-scu-PA treatment).

2.2.1.3

Left circumflex coronary artery (LCX) experiments

(diagram 11)

Aim:

to test taprostene cotherapy with r-scu-PA in a model established after conclusion of the previous two experiments.

4 groups were tested:

group I - LCX thrombosis-vehicle treated controls

group II - r-scu-PA infusion started 90 minutes after the onset LCX thrombotic occlusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$; i.v.; 30 minute duration)

group III- r-scu-PA infusion as above (group II) together with taprostene at a low infusion rate (0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.; 120 min duration)

group IV - r-scu-PA infusion (as in group II) together with taprostene at a higher infusion rate (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.; 120 min duration)

Summary of Experimental Protocols:

Dog coronary thrombolysis

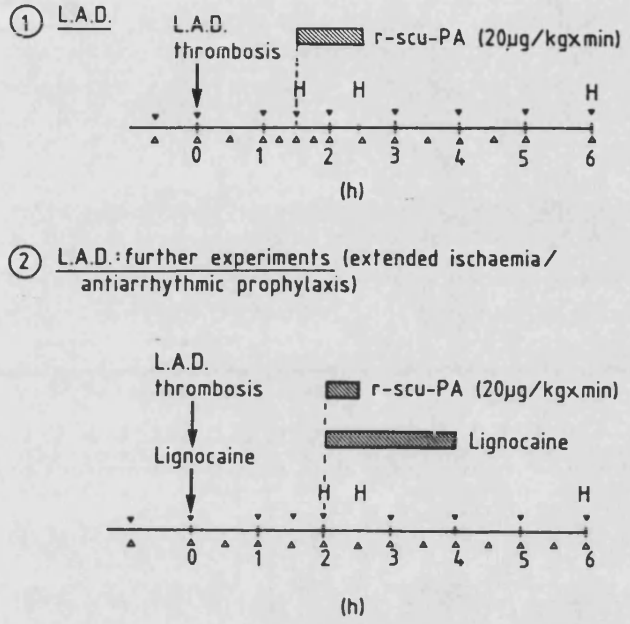
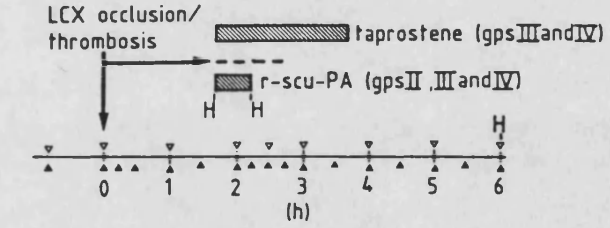


Diagram 10

Dog LCX thrombosis



▾ -venous blood samples
▴ -haemodynamic recordings
H -heparin bolus (1000U. i.v.)

Diagram 11

Diagrams 10 and 11: Summary of experimental protocols used in canine coronary artery (LAD and LCX) thrombolysis experiments

Surgical preparation and instrumentation of canine models

Adult beagle dogs (both sexes, 13.6-19.8 kg), kept indoors and maintained on a 12 hour light/dark cycle had their normal chow diet withdrawn 18 hours prior to operation. Anaesthesia was induced by thiopentone (10 mg·kg^{-1} i.v.), and maintained with pentobarbitone as required to abolish the corneal reflex. Body temperature was maintained at 39.0 ± 1.0 °C using a heating pad. Abdominal aortic blood pressure (BP) was measured by inserting a polythene catheter, connected to a precalibrated pressure transducer (Statham P23Db), into the femoral artery. Mean arterial blood pressure was calculated as MABP = diastolic blood pressure + (0.42 x blood pressure amplitude). Left ventricular end diastolic (LVEDP) and peak-pressure (LVPP) were measured using a tip manometer (Millar PC 350) inserted via the left carotid artery. Left ventricular contractility, expressed as the maximum rate of increase in systolic pressure (+dP/dt), was calculated on a Hellige analogue computer. Cannulation of the femoral and jugular veins enabled drug administration and blood sampling respectively. Subcutaneous needles were implanted to record a Lead II electrocardiogram (ECG). A tracheal tube with an inflatable collar was inserted enabling positive-pressure respiration (tidal volume 15-20 ml·kg^{-1}, frequency 10 min^{-1}). Respiration was adjusted continually so that blood gases were maintained in the physiological range (Po_2 100-140 mm Hg, Pco_2 32-40 mm Hg). Haemodynamic parameters and ECG's were recorded on paper using a multichannel recorder (Hellige 330 P).

A fifth intercostal thoracotomy and suspension of the heart in its pericardium provided an anterior aspect. Starting 1-2 cm from its origin, a 3 cm segment of the coronary artery (LAD or LCX) was prepared by gently separating the overlying connective tissue. Two snares were loosely placed around the artery, 1 cm apart. All of the small side branches lying between the snares were ligated except for 1 which was cannulated for thrombin introduction later. An electromagnetic flow probe was placed around the artery proximal to the snares.

Thrombus formation

After more than 30 minutes equilibration time the snares were drawn and the artery segment lying in between was crushed repeatedly with blunt forceps in order to damage the endothelium. Thrombin (10 units, human) and fresh blood were then injected into the occluded artery segment via the previously cannulated small side branch. After allowing the thrombus to mature for approximately 50 minutes the upper snare was released followed by the distal snare after 5 - 10 minutes. Thrombotic occlusion was confirmed after release of both snares by a zero flow reading, the dark appearance locally and by the absence of a sudden burst of arrhythmic activity.

LAD experiments (1 + 2) were carried out "open label", whereas LCX experiments (3b + 3c) were carried out blind and randomized. After fulfilling the inclusion criteria: stable thrombotic occlusion, ECG S-T segment elevation ($\geq$ 0.2 mV, lead II) and marked visible cyanosis of the ischaemic region, the animals were randomised to

receive treatment: The treatment regimens, as well as the administration of heparin (1000 units i.v. bolus) and the venous blood sampling times are shown in diagrams 10 and 11.

Plasma assays

Venous blood samples were anticoagulated as specified in the various assays (section 2.2.4) and centrifuged immediately (2000 g, 15 minutes, 20-25 °C). Plasma aliquots for the creatine kinase (CK), α-2-antiplasmin (AP) and plasminogen assays were carefully aspirated from the supernatant, and stored at -30°C for up to 2 months before use. Fibrinogen determination was carried out directly on fresh plasma.

Estimation of relative infarct size

Six hours after the intial coronary artery occlusion heparin (1000 units, i.v. bolus) was given followed by a massive overdose of pentobarbital. The hearts were excised and immediately treated as described: A dual-perfusion method, adopted from Lucchesi, Romson, and Jolly (1984) (Diagram 12) was used to simultaneously stain: a) the myocardial region supplied by the (previously) occluded coronary artery i.e. the anatomic area at risk of infarction (AaR) and b) the necrotic region or infarcted area (I). Triphenyl tetrazolium chloride (TTC, 1.5 % in 70 mmol·l^{-1} phosphate buffer, pH 7.0, 37°C) was perfused into the coronary artery using a cannula inserted just distal to the lower snare. Evan's blue (1 % in physiological saline, 37°C) was perfused into the remaining coronary circulation following cannulation of the aorta.

Perfusion pressure was maintained at 120 mm Hg for 5 minutes. The right ventricle free wall was cut away and the left ventricle sliced perpendicularly to its axis at 1 cm intervals. The slices were photographed (Kodak Ektachrome 50, Tungsten light). Area not stained with Evan's blue was assumed to have been at risk (AaR). The heart slices were placed in TTC solution (37°C) for 5 min to check that TTC perfusion of the myocardium was thorough. Tissue not staining deep red with TTC, and confined to within the AaR, was classified as infarcted (I) (Vivaldi, Kloner and Schoen 1985). Computer assisted planimetric assessment of photographs enabled the following to be estimated for each heart: the proportion of the left ventricle myocardium directly perfused by the artery in question (% AaR/LV) and the proportion of the risk area and left ventricle that were necrotic, as detected by TTC, (% I/AaR and % I/LV respectively).

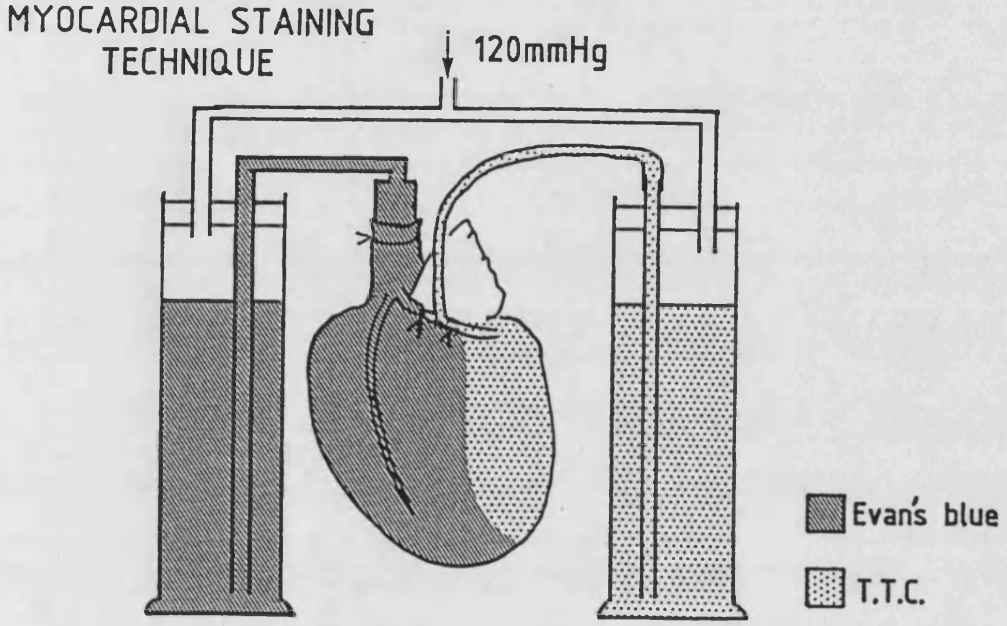


Diagram 12

Balanced pressure myocardial staining technique
Coronary artery perfusion ex-vivo with TTC and Evan's blue solutions to determine infarct size and anatomic area at risk (AaR)

Arrhythmias

The number of ectopic beats per 20 consecutive beats was recorded at 4 separate times for each time interval specified:

-1 h to 0 h, 0 h to 1 h, 1 h to 1.7 h, 1.7 h to 3 h, 3 h to 4 h, 4 h to 5 h, 5 h to 6 h

2.2.2 *Experiments to study the separate and combined effects of taprostene and r-scu-PA on blood flow through a carotid artery stenosis in rabbits*

Male white New Zealand rabbits (1.8 - 3.1 kg) were anaesthetised with pentobarbitone (30 $mg \cdot kg^{-1}$ i.v.). An electromagnetic flow probe (Hellige, FRG) was placed around the right carotid artery. Blood flow was recorded continuously both as the instantaneous flow rate, and interval mean flow rate over the specified intervals. The latter was obtained by electronically converting the flow probe voltage to counting rate. Total counts for a specified period were converted to milliliters blood using a calibration curve, and mean flow rate over the specified interval obtained by dividing interval blood flow (ml) by interval duration (min). Both flow rate values were converted to a standard body weight of 1 kg by dividing by the animals weight. The left and right femoral veins were cannulated for drug administration and blood sampling. Arterial blood pressure was recorded using a pressure transducer (Statham P23D) after cannulation of the right femoral artery with saline-filled polythene tubing.

The pretreatment parameters were recorded, including plasma fibrinogen, platelet aggregation ex vivo, blood pressure and carotid artery flow.

The animals were then allocated randomly into 4 groups to receive:

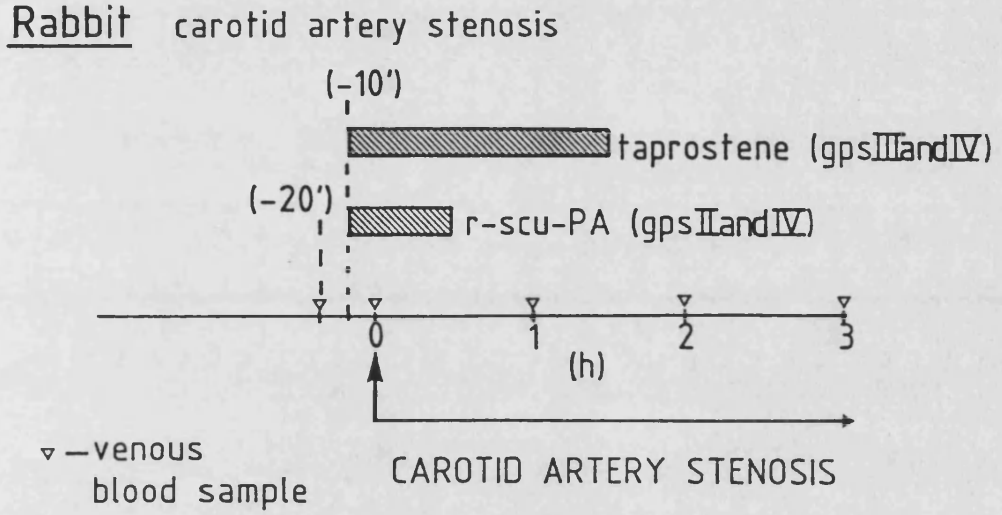
group I: saline (n=6),

group II: r-scu-PA (21.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v. for 40 min, n=5),

group III: taprostene (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v. for 100 min, n=5)

group IV: taprostene and r-scu-PA cotreatment according to their separate protocols (groups II and III) (n=5) (see diagram 13).

Infusion rates were low (< 0.5 ml/h), and withdrawn blood samples were replaced with an equal volume of saline. Ten minutes after starting the infusions a critical stenosis was introduced by implanting a 12 mm segment of polythene tubing (internal diameter 0.86 mm) in the carotid artery. This was done after clamping a 2 cm artery segment with 2 artery clamps. The polythene tubing was inserted in the occluded artery segment through an oblique incision and was fixed in position by ligatures at both ends. The flow probe was calibrated to read 0 ml/min and the artery clamps removed (t = 0). In no instance was the carotid blood flow interrupted for longer than 30 seconds. Blood flow readings and venous samples were taken at regular intervals according to diagram 13. The experiment was terminated at 180 minutes after implantation of the stenosis. A cyclical flow variation (CFR) event was defined as a rapid increase in flow rate (> 0.5 ml/min within 30 seconds), followed by a renewed decrease in flow rate. These CFR were clearly visible on the flow rate paper traces.



Continuous haemodynamic measurement

-carotid -flow rate

-interval-flow volume

-MABP

Diagram 13

Study protocol used to examine the effect of r-scuPA and/or taprostene infusions on thrombosis in a carotid artery stenosis model in rabbits.

2.2.3 *Experiments to study the effects of taprostene infusion on endogenous fibrinolytic activity in man*

Subjects:

Six male volunteers (19-45 years old, 60-90 kg, Broca index 80-120) took part in the study after giving their informed consent and after having fulfilled the test inclusion criteria:

(1) Perfect health, certified after recent examination by an external consultant internist.

(2) No exposure to the agents specified during the preceeding periods:

- *hepatic enzyme inducing drugs: 3 months*
- *drugs affecting blood coagulation or fibrinolysis: 1 month*
- *drugs affecting platelet aggregation: 1 month*
- *alcohol: 7 days*

Study design:

A randomised, single blind, controlled, cross-over study regimen was used. Each of the volunteers received only one of the three treatments per experimental day. A 7-day interval between experimental days was observed for each volunteer and each volunteer eventually received each of the three treatments (Diagram 14):

- *placebo infusion (buffer, i.v. infusion of 2 hours duration)*
- *low dose taprostene infusion (80 µg over 2 hours i.v.)*
- *high dose taprostene infusion (400 µg over 2 hours i.v)*

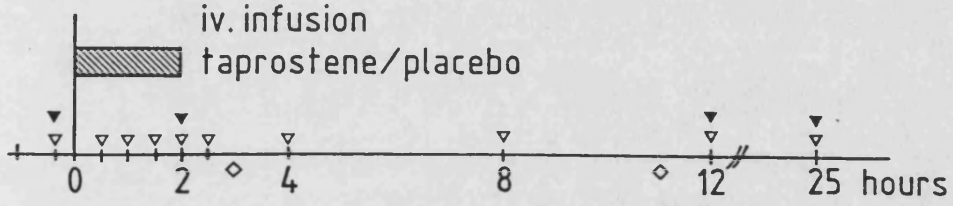
Before starting the experiment, the volunteers assumed a supine position, which was maintained for the duration of the infusion. Pretreatment venous blood samples, brachial blood pressure and heart rate readings were taken. The identity of the infusion was unknown to the volunteers and to the haematology laboratory. Blood samples, taken at the times specified in diagram 14, were anticoagulated as specified in section 2.2.4 (in vitro assays) and were assayed for:

- *plasma fibrinogen*
- *plasma α-2-antiplasmin*
- *plasminogen activator inhibitor 1 (PAI-1)*
- *platelet factor 4 (PF4)*
- *euglobulin clot lysis time (ECLT)*

The volunteers had fasted overnight until 1 hour after the end of the infusion, when they had a light lunch. A small evening meal was eaten 8 hours after this at 19.00 hours.

After termination of the infusion the volunteers assumed upright positions and were permitted to walk. Subsequent blood samples were taken after a few minutes in a supine position. The last blood samples were taken 23 hours after termination of the 2 hour infusion on each experimental "day".

Taprostene infusion study in man:



▼ - Fibrinogen, α_2AP assays
▽ - ECLT, PF_4, PAI assays
◇ - meals

Diagram 14
Protocol for taprostene study in healthy male volunteers
α_2AP = α_2 antiplasmin, ECLT = euglobulin clot lysis time,
PF4 = platelet factor 4, PAI = plasminogen activator inhibitor

2.2.4 In vitro Assays

2.2.4.1

Fibrinogen

Principle:

When an excess of thrombin is added to diluted citrated plasma, the time to the appearance of a fibrin clot is inversely proportional to the fibrinogen concentration (Clauss 1957).

Procedure:

Venous blood was citrated (9 parts + 1 part Na-citrate, 0.11 M) and centrifuged (15 min, 2000 g, 20-25°C). The resulting fresh citrated plasma was diluted (1 part plasma + 9 parts Owren's veronal buffer, pH 7.35). Aliquots (200 µl) of diluted plasma were incubated at 37 °C for 1 minute in an automatic 2 channel coagulometer. Human thrombin (100 µl, 100 NIH units/ml) was added and the time to coagulation recorded. For each batch of thrombin a calibration curve was obtained using reference human fibrinogen, from which plasma sample concentrations were calculated in mg/100 ml. For each animal the preischaemic plasma fibrinogen concentrations served as the 100 % value from which subsequent values were calculated (%). Duplicate assays were performed.

Comments:

The measurement of fibrinogen with this method was possible in the presence of heparin (dog studies) due to the supplier's addition of a heparin inhibitor to the human thrombin reagent.

The addition of plasmin inhibitors, e.g. Aprotinin, was not neccessary in order to prevent in vitro fibrinogenolysis due to r-scu-PA as samples were assayed immediately after collection.

2.2.4.2

$\underline{\alpha_2\text{-antiplasmin (AP)}}$

Principle:

*When an excess of plasmin (Pli) is added to diluted plasma an inactive 1 : 1 stoichiometric complex plasmin : α_2-antiplasmin complex is very rapidly formed. The remaining free plasmin activity can be measured photometrically by the rate of paranitroaniline (pNA) release from the peptide substrate S-2251 (H-D-Val-leu-lys-pNA*2HCl) (Teger-Nilson, Friberger and Gyzander 1977; Friberger 1982).*

(1) $AP + Pli_{(xs)} \longrightarrow AP : Pli + Pli$

(2) $S\text{-}2251 \xrightarrow{Pli} peptide + pNA$

Procedure:

Citrated plasma was diluted (1 : 3 in Tris buffer 0.05 M, pH 7.4, I=0.15) and aliquots (300 µl) were equilibrated at 37°C for 2 to 6 minutes. Human plasmin (100 µl aliquots, 0.3 CU/ml in 2 mM HCl containing 50 % v/v glycerol) was added, mixed, and incubated for exactly 20 seconds. Peptide substrate S-2251 (100 µl, 3.5 mM in distilled water) was added and the initial rate of change of extinction (ΔE/min) measured at 405 nm. Total plasmin activity was recorded for each series by substituting Tris buffer for diluted plasma (=0% antiplasmin, R_o).

Duplicate assays were performed. For human plasma samples a calibration curve was obtained by serial dilution of a reference human plasma pool from 15 healthy volunteers (9 male, 6 female, ages 21 - 45). Undiluted pool plasma was taken as 100 % antiplasmin activity (R_{100}). In the dog studies the pretreated antiplasmin activity served as the 100 % value (R_{100}) for each dog. The percentage of AP was calculated using the formula:

$$x(\%) = \frac{R_0 - R_x}{R_0 - R_{100}} \times 100$$

where R_x is the activity (ΔE/min) of the sample tested

x is the % antiplasmin of the sample tested

R_{100} is the activity of - the plasma pool (human study)

- the preischaemic plasma sample

(dog study)

Comments:

Antiplasmin activity in plasma reflects the function of several inhibitors including α_2*-macroglobulin and* α_1*-antitrypsin. The initial rate of inhibition of plasmin is however widely regarded as being* α_2*-antiplasmin-specific, due to its extremely rapid effect (*$t_{1/2}$ *inhibition = 0.1 sec (Friberger 1982)).*

2.2.4.3

Plasminogen

Principle:

When an excess of urokinase or streptokinase is added to diluted plasma containing plasminogen a complex is formed which itself catalyses the amidolytic liberation of the chromophore pNA from the substrate S-2251. The rate of pNA release is proportional to the amount of plasminogen in the sample (Friberger et al. 1978; Scully and Kakkar 1978).

Procedure:

Citrated plasma was diluted (1 : 61 with 50 mM Tris buffer, pH 8.8, containing 8.3 mM E -aminocaproic acid) and aliquots (100 µl) were incubated with recombinant light chain urokinase (clUK, 100 µl, 3,500 Ploug units/ml) at 37°C for 3 minutes. Tris buffer (40 µl, 1M, pH 10.6) was added followed by the chromogenic substrate S-2251, (300 µl, 1 mM in Tris/maleate buffer 67 mM, pH 6.0). The initial rate of reaction (ΔE /min) was recorded photometrically at 405 nm and 37°C. Blanks were obtained by substituting diluted plasma samples with Tris buffer. Duplicate assays were performed. The pretreatment value of plasminogen served as the 100 % value for each dog, from which the subsequent values were calculated.

2.2.4.4

Creatine Kinase (CK-NAC)

Principle:

Creatine kinase (CK) catalyses the formation of adenosine triphosphate (ATP) and creatine from adenosine diphosphate (ADP) and creatine phosphate (CP):

$$ADP + CP \xrightarrow{CK} ATP + creatine$$

ATP thus formed is linked to the reduction of NADP, which can be followed photometrically at 334 nm.
(Rosalki 1967).

$$Glucose + ATP \xrightarrow{Hexokinase} Glucose\text{-}6\text{-}P + ADP$$

$$Glucose\text{-}6\text{-}P + NADP^{+} \xrightarrow{G6P\text{-}DH} 6\text{-}phosphogluconate + NADPH$$

Procedure:

Venous blood (9 parts) was added to heparin (1 part 1000 U/ml) and centrifuged (2000 g, 15 min, 25°C). Aliquots (20 µl) of heparinized plasma were added to the CK reagent solution (500 µl) and mixed. After 3 minutes incubation at 25°C the rate of reaction (ΔE/min, 334 nm) was recorded for 3 minutes. Plasma samples exhibiting CK activity of more than 0.2 ΔE/min were diluted 1 : 10 with physiological saline. Duplicate assays were performed.

Plasma CK activity (U/l) was calculated using the formula provided by the test kit suppliers:

$$U/l\ (25°C) = 4207 \times \Delta E_{334\ nm}/min$$

Comments:

3 or more CK-isoenzymes exist: CK-MM, CK-BB and CK-MB. Human myocardium contains about 70 % MM and 30 % MB. CK-BB and CK-MM occur mainly in the brain and skeletal muscle respectively. Loss of cell integrity results in the elution by, and appearance in, blood of CK activity. In man, CK-MB activity can be estimated by subtracting anti-CK-MM antisera treated plasma activity from total plasma CK activity (Wagner, Roe and Limberd 1973). This technique has not been modified for animal studies, but the contribution by non-cardiac sources (e.g. intercostal muscle enzyme release due to thoracotomy) can be estimated by blood sampling after surgical preparation and prior to myocardial ischaemia.

2.2.4.5

Euglobulin clot lysis time (ECLT)

Principle:

When cold diluted plasma is acidified to pH 5.9 the euglobulin fraction, containing fibrinogen, plasminogen and plasminogen activators, is precipitated. Most of the inhibitors of plasminogen activation remain in solution. The precipitate can be redissolved and coagulated with excess thrombin. The rate of autolysis of the clot at 37°C is widely considered as an indicator of the fibrinolytic activity of the sample (Giddings 1988).

Procedure:

Aliquots (300 µl) of fresh citrated plasma were diluted in glass tubes with distilled water (4.6 ml, 4°C) and acidified with acetic acid (100 µl, 0.75 % v/v) to pH 5.9. The tubes were inverted then cooled for 15 minutes at 4°C, followed by centrifugation (1500 g for 5 minutes) and aspiration of the supernatant. The tube walls were carefully swabbed and the pellet redissolved in saline phosphate buffer (300 µl, pH 7.2). The euglobulin solution was transferred to sealed plastic cuvettes, thrombin (bovine, 300 µl of 10 NIH units/ml) added and the cuvettes mixed rapidly by vortexing. The cuvettes were placed in a photometer maintained at 37°C and the absorption at 578 nm measured at 10 minute intervals over the following 9 hours. Triplicate assays were performed. The lysis time was recorded as the time required for the photometric absorption to decrease to 20 % of the stable terminal absorption value which corresponded to visual detection of complete lysis. The endogenous fibrinolytic rates were calculated as the reciprocal of the lysis times (in minutes).

Comments:

Fast acting plasminogen activator inhibitor (PAI-I) is precipitated in the euglobulin fraction along with minimal C1 inactivator. The reciprocal of the clot lysis time (1/ECLT) quantifies therefore the composite effect of plasminogen activators and inhibitors (Kluft and Brackman 1975; Wijngaards 1979).

CTAD plasma

Special commercially avalaible tubes (Diatube-H) were employed for collecting blood samples to avoid ex vivo platelet activation. Free flowing venous blood (4.5 ml) was collected in precooled CTAD tubes containing antiplatelet agents: citrate, theophylline, adenosine and dipyridamole. The tubes were kept on ice for 15 minutes, then centrifuged at 2000 g for 15 minutes at 6°C. The platelet poor plasma was immediately aspirated from the middle of the supernatant column and stored at -30°C for less than 2 weeks. Before use the plasma samples were thawed quickly at 37°C to prevent cryoprecipitation, then mixed by vortexing and kept at 2°C. CTAD plasma samples were used in the determination of platelet factor 4 (PF4) and plasminogen activator inhibitor (PAI-1).

2.2.4.6

Plasminogen activator inhibitor-1 (PAI-1) assay

Principle:

When a fixed amount of tPA is added in excess to plasma an inactive PAI-1•tPA complex is formed (1). The excess tPA can be measured, after complete stimulation, by the amount of plasmin formed from plasminogen (2). Plasmin activity is measured spectrophotometrically with the peptide substrate S-2251 (3).

$$PAI\text{-}1 + tPA_{(xs)} \longrightarrow tPA{\cdot}PAI\text{-}1 + tPA \qquad (1)$$

$$Plasminogen \xrightarrow{tPA + tPA\ stimulator} Plasmin \qquad (2)$$

$$Plasmin + S\text{-}2251 \longrightarrow amidolysis\ product + chromophore\ pNA \qquad (3)$$

Procedure

The samples and standards were prepared as follows:

Step	STANDARDS		SAMPLES
	0 AU/ml	40 AU/ml	(CTAD plasma)
1	25 µl 40 IU/ml t-PA	25 µl Tris buffer	25 µl 40 IU/ml t-PA
2	25 µl PAI-1-depleted plasma	25 µl PAI-1-depleted plasma	25 µl CTAD plasma sample
3	Incubate 10 minutes at 20 - 25°C		
4	4.0 ml sterile water		
5	Mix		

By mixing corresponding volumes of the standards 0 and 40 AU/ml, further standards were obtained: 30, 20 and 10 AU/ml PAI-1. Aliquots (100 µl) of the resulting diluted samples or standards were transferred to 96-well microtitre plates (flat bottom, Greiner, FRG) followed by aliquots (100 µl) of a freshly prepared plasminogen/chromogenic substrate solution (composition of the latter: human plasminogen: 2 CU/ml, Kabi chromogenic substrate S-2251: 0.76 mM, Tris: 0.04 M, Tween: 0.008 %, pH 8.3).
After this, 50 µl aliquots of t-PA stimulator solution (human fibrin(ogen) fragments, 0.67 mg/ml in 0.04 M Tris) were added and the microplates incubated at 37°C on a circular-displacement mixing table. After a predetermined optimal time of 90 minutes the incubations were stopped with acetic acid (50 µl aliquots of 20 % v/v) and the absorbance at 405 nm read against distilled water. PAI-1 activities in the plasma samples were calculated from the standard curves, one of which was run per microplate. All assays were carried out in triplicate.

Comments:
Platelets contain a significant proportion of the PAI-1 in blood (Bachmann 1987) and ex vivo platelet activation leads to elevated PAI-I values (Kruithof 1986). This source of error would additionally be subject to a one-sided bias if antiplatelet drug-(taprostene) treated groups are compared with controls. A meticulous blood sampling/ treatment regime in addition to ex vivo antiplatelet treatment (CTAD) reduces the degree of ex vivo activation. A platelet-release marker assay (Platelet factor 4, PF4) was carried out in parallel with the PAI-1 assay and plasma samples with PF4 concentrations greater than the arbitrary cut off value of

50 IU/ml were rejected as they were assumed to have undergone an ex vivo platelet release reaction.

2.2.4.7

Platelet Factor 4 (PF4) assay

Principle:

PF4 has several antigenic determinants allowing a quantitative enzyme-immunoassay (ELISA) based on the sandwich principle.

Procedure:

Micro-ELISA plates (Nunc. Immunoplate I, Denmark), coated overnight with Fab' anti-PF4 antibody solution, were washed and sequentially incubated with a) CTAD plasma samples or standard PF4, then b) anti-PF4 antibody-peroxidase conjugate, both for 1 hour at 25°C with washing between incubations.

Orthophenylene diamine (OPD) substrate was then added and incubated at 25°C . After the predetermined optimal reaction time had elapsed the reaction was stopped with hydrochloric acid (1 M). The absorbence was read at 492 nm within 1 hour. PF4 concentrations in the CTAD plasma samples were then calculated using a calibration curve run in parallel, employing standard PF4 supplied. All determinations were done in triplicate.

Comments:

PF4, like ß-thromboglobulin, is a platelet specific substance with a distribution index (platelet/plasma) of 20,000 and an elevated level in plasma is therefore a sensitive and specific indicator of

platelet activation. The normal healthy human plasma value is 0-5 IU/ml (Zahavi, Jones, Leyton et al. 1980; Dawes, Smith and Pepper 1978; Kaplan and Owen 1981).

2.2.4.8

Platelet aggregation

Venous blood was citrated (1 : 9 with 0.11 M Sodium citrate) and centrifuged for 10 minutes at 200 g (22°C). The supernatant or platelet rich plasma (PRP) was gently aspirated and the pellet centrifuged again for 10 minutes at 1,800 g (22°C) to obtain a platelet poor plasma (PPP) supernatant. PRP aliquots (300 µl), at room temperature, were prewarmed to 37°C in siliconized glass tubes for 3 minutes and the light transmission recorded (T_0). Collagen (native equine tendon) was then added in 25 µl of suspension to give end concentrations of 1, 2 and 5 µg/ml collagen. The change in light transmission was followed for 10 minutes and % aggregation (%A) calculated using the formula:

$$\% A = \frac{T_0 - T_x}{T_0 - T_{100}}$$

T_{100} = absolute light transmission of PPP

T_0 = absolute light transmission of unstimulated PPP

T_x = absolute light transmission of plateau after collagen stimulation of PRP.

Single assays were performed.

3 **Results**

3.1 LAD artery thrombolysis experiments in dogs

Four of the 14 dogs entering this study were not assessed for the following reasons:

- 2 dogs succumbed to ventricular fibrillation at 7 and 14 minutes respectively after the onset of abrupt coronary occlusion (snares drawn). These animals had not yet been allocated to an experimental group at this time.
- 2 dogs, both in the r-scu-PA treated group (group c) underwent ventricular fibrillation, both 30 - 40 minutes after the onset of ischaemia and both temporally associated with the onset of LAD reperfusion as detected by the flow probe.

The remaining 10 dogs completing the study were grouped accordingly (see Diagram 10):

group a) 6-hour LAD ligation (n = 3)

group b) untreated LAD thrombosis (n = 3)

group c) LAD thrombosis, then after 90 min occlusion r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 60 min duration, n = 4)

3.1.1 LAD artery patency/Dog

All six animals treated with r-scu-PA underwent successful LAD recanalisation; 2 of these however were excluded from the analysis after ventricular fibrillation (see above). The mean time to recanalisation after commencing r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 60 minutes) was 25 ± 5 minutes ($\bar{x}$ ± S.E.M.), hence a mean duration of ischaemia of 115 minutes in this group (group c). In the untreated thrombus group (group b) a zero LAD-flow reading was observed for the duration of the experiment demonstrating stability of thrombi formed by this method.

3.1.2 Haemodynamic parameters/Dog LAD thrombolysis

Heart rate/LAD/Dog

The heart rates were reproducibly high (group means (± S.E.M.) groups a and b combined = 177 ± 10 min^{-1} and group c 172 ± 15 min^{-1} (beats/min)) prior to ischaemia. After LAD occlusion there was a general trend to increased heart rates which continued over the 6 hour period in both the continuous occlusion groups (a and b) and the r-scu-PA treated group (group c) although at various scattered times before and after r-scu-PA infusion the heart rate in this group was significantly lower, but this was not definitely attributable to r-scu-PA infusion as it occurred before treatment started (table 1).

Mean Arterial Blood Pressure (MABP)/LAD (1)/Dog

The initial preischaemic values of the mean arterial blood pressure (MABP) were similar in the 3 treatment groups ($\bar{x} \pm$ S.E.M.):

a) 6 hour LAD ligature ($\bar{x}$ = 110 mm Hg, n = 3),

b) 6 hour LAD thrombosis ($\bar{x}$ = 102 mm Hg, n = 3),

c) r-scu-PA thrombolysis (96 ± 9 mm Hg, n = 4)

(diagram 15, table 1).

The MABP fell moderately directly after occlusion of the LAD to approximately 85 mm Hg in all groups, but by 30 minutes post occlusion had recovered slightly. This effect was very similar in the 3 groups prior to the different treatments. In group (c) r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.), starting 90 minutes after thrombotic occlusion was associated with a continuous fall in MABP to approx. 60 mm Hg at the end of the 60 minute infusion.

The MABP stabilized at about 50 mm Hg, 1 hour after the r-scu-PA infusion had been terminated until the end of the experiment which was 6 hours after the initial onset of ischaemia. In the ligature group (group a) and untreated thrombus group (group b) the MABP decreased more slowly than in the r-scu-PA group (group c), to a 6 hour value of approximately 80 mm Hg in both continuous occlusion groups (groups a and b). Application of the Student's 2-tail t-test showed statistically lower MABP ($p>0.05$) in the r-scu-PA group (group c) starting immediately after completion of the infusion (t = 2.5 h) and for approx. 2 h duration thereafter (up to t = 4.5 h). Beyond this time (t = 4.5 h) the difference in MABP between the pooled continuous occlusion groups (groups a and b) and the r-scu-PA treated group was no longer significantly different.

Left ventricular contractility (+dP/dt)/LAD/Dog

The means of the +dP/dt values (+dP/dt = maximum rate of increase in left ventricular pressure) varied moderately between the 3 groups prior to ischaemia (mean of groups a and b (± S.E.M.) = 1816 ± 249 mm Hg s^{-1} and group c = 1675 ± 249 mm Hg s^{-1}). They converged however within 1 hour of occlusion as the contractility decreased. Thereafter the contractility waned slightly and similarly in both 6 hour occlusion groups (a and b). In the r-scu-PA infusion group (c) the contractility decreased moderately below the values seen in the continuous occlusion groups ($p<0.05$) during the 2 hours of thrombolytic induced reperfusion which the contractility tended to increase slowly towards the values recorded in the continuous occlusion groups (diagram 16, table 1).

Left Ventricular Peak (Systolic) (LVPP) and End Diastolic (LVEDP) Pressure/LAD (1)/Dog

LVPP:

Preischaemic LVPP values were similar for the 3 groups (range of group means : 117 mm Hg (group c) to 136 mm Hg (group b). Continuous LAD occlusion (groups a and b) was associated with a biphasic decrease in peak pressure developed; an initial slight fall in peak pressure seen in all 3 groups (≈ 15 %) within minutes of LAD occlusion, followed by a slower decrease in LVPP over the following 6 hours seen in the 2 continuous occlusion groups (diagram 17, table 1).

r-scu-PA infusion did not significantly affect the peak ventricular pressure over the 6 hours observed when compared to the other 2 6-hour-occlusion groups after pooling them (groups a and b).

In all groups the terminal (t = 6 h) peak ventricular pressure values were similar and approx. 25 % less than the preischaemic values (diagram 17, table 1). There was no significant difference (p > 0.05, Student's t-test, 2-tail, unpaired data) between r-scu-PA treated (group c) and untreated (groups a and b) groups.

LVEDP

No major change in the minimal ventricular pressure was seen during the 6 hours observed either as a result of ischaemia (thrombus or ligation) or thrombolytic treatment with r-scu-PA after thrombosis (diagram 17, table 1).

3.1.3 Haemostatic parameters/Dog LAD thrombolysis

α-2-Antiplasmin (AP)/LAD/Dog

No marked change of plasma AP activity in the groups without r-scu-PA treatment (groups a and b) was observed over the 6 hours studied. In contrast, in the r-scu-PA treated group (group c) a marked decrease in AP was observed:
A nadir of the group mean AP activity (≈ 50 % of preischaemic value) occurred 3.5 hours after starting the 1 hour infusion of r-scu-PA. At this time (t = 5 hours), and 1 hour before and after, the AP activities were significantly depressed (p < 0.05) compared to r-scu-PA untreated controls (groups (a) and (b)) (diagram 18, table 2).

Plasminogen/LAD/Dog

Plasma plasminogen levels remained roughly constant over the 6 hour period in both continuous occlusion groups (a+b). In the r-scu-PA treated group (group c) plasma plasminogen decreased to a mean of 66 % of the pretreatment level 2.5 hours after completing the 1 hour infusion of r-scu-PA (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$). No significant difference ($p > 0.05$) between the r-scu-PA group (c) and the pooled groups (groups a and b) (diagram 19, table 2) was recorded however.

Fibrinogen/LAD/Dog

The plasma fibrinogen concentrations remained constant over the 6 hour period in both continuous LAD occlusion groups (a) and (b). A mean fall in plasma fibrinogen of 30% compared to pretreatment values was recorded 30 min after completing the r-scu-PA infusion. Thereafter the fibrinogen levels started to recover slowly. This decrease in fibrinogen accompanying the infusion of r-scu-PA (over 1 hour) was significantly ($p<0.05$ Student's 2-tail t-test) different from the pooled levels from the 2 untreated groups (a) and (b) (diagram 20, table 2).

3.1.4 *Infarct size parameters including creatine kinase activity/ Dog LAD thrombolysis*

Creatine Kinase/LAD/Dog

In both groups in which the LAD was permanently occluded (groups a and b) a very similar slow but continuous rise in plasma CK activity was measured, reaching 600 - 800 U/ml after 6 hours. In r-scu-PA treated animals (group (c)) a precipitous increase in plasma CK activity accompanied the recanalisation of the thrombus-occluded LAD artery. In this group the peak mean CK activity (2665 183 U/ml ($\bar{x}$ ± S.E.M.)) was recorded 2.5 hours after starting the 1 hour infusion of r-scu-PA, after which a slight decrease in plasma CK activity occurred. The very high mean CK activity in the r-scu-PA treated animals was significantly greater ($p < 0.01$ at t=4 h) than that of the combined continuous occlusion groups (groups a and b).

Infarct size estimation/LAD/Dog

Proportion of LAD perfused myocardium infarcted at 6 hours (% I/AaR)

Myocardium not stained deep red with TTC was classified as infarcted. In the ligated and thrombus occluded groups (a and b) a similar proportion (≈ 50%) of the LAD artery bed was unstained therefore necrotic at 6 hours I/AaR (51.7 ± 15 % and 47.6 ± 5 % respectively; range 24 to 74 %). r-scu-PA infusion after 90 minutes of LAD thrombotic occlusion resulted in a relative infarct size of 9.3 ± 2 % at 6 hours, which was significantly ($p < 0.01$, Student's t-

test, 2-tail) smaller than that of the pooled continuous occlusion groups (groups a and b)(diagram 22, table 3). The proportion of left ventricular myocardium supplied by the LAD (% AaR/LV) was similar in all groups (18.9 ± 2.5 %: 6 hour ligature (n=3); 14.6 ± 1.5% 6 hour thrombotic occlusion (n=3); 13.2 ± 2.2 % (n=4): r-scu-PA group). The range of values (all groups) for AaR/LV was from 7.9% to 23.8%. The LAD therefore directly perfuses approximately 1/6 of the left ventricular myocardial mass (diagram 22, table 3).

3.1.5 <u>Further LAD experiments with prolonged duration of ischaemia and antiarrhythmic prophylaxis/Dog</u> (Methods, section 2.2.1.2)

r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.) started after 120 minutes of LAD thrombotic occlusion resulted in recanalization in all animals (n = 4). The relative infarct size (%I/AaR) 6 hours after the initial occlusion was found to be 18.2 ± 7.2 %. The LAD-perfused bed was 28.6 ± 0.5 % of the left ventricular myocardium (%AaR/LV). Haemodynamic and haemostatic parameters were essentially similar to those of the corresponding treatment groups in the previous experiment (data not given).

Summary of Dog LAD thrombolysis experiments

To summarize the results of the LAD experiments ligation or stable thrombotic occlusion of the LAD for 6 hours produced comparable effects on the haemostatic and haemodynamic variables (diagrams 15, 16, 17). The infarct sizes were also similar in both groups of animals (≈ 50% of the anatomical risk area) (diagram 22). Thrombolytic treatment with r-scuPA (20 $\mu g \cdot kg^{-1} \cdot min^{1}$, 60 min) starting 90 min after stable LAD thrombosis produced divergent effects on these variables however. MABP and left ventricular contractility were reduced (diagrams 15 and 16) and limited plasmin activation was evident from a moderate decrease in plasma levels of α_2-antiplasmin, plasminogen and fibrinogen (diagrams 18, 19, 20). Successful thrombolysis occurred in all animals treated with r-scuPA, however 2 of 6 animals underwent ventricular fibrillation within 1 hour of coronary reperfusion. Infarct size expressed as a % of the anatomic "risk area" was reduced in this treatment group $(9.3 \pm 1.6\%)$ relative to the dogs with 6 hour continuous LAD occlusion of the anatomic risk area (diagram 22). The rapid rise and early peak of CK activity in plasma is further evidence of early reperfusion in the r-scuPA treated animals (diagram 21). Further studies were performed using the LCX coronary artery as the mortality following LAD occlusion and reperfusion proved to be unacceptably high.

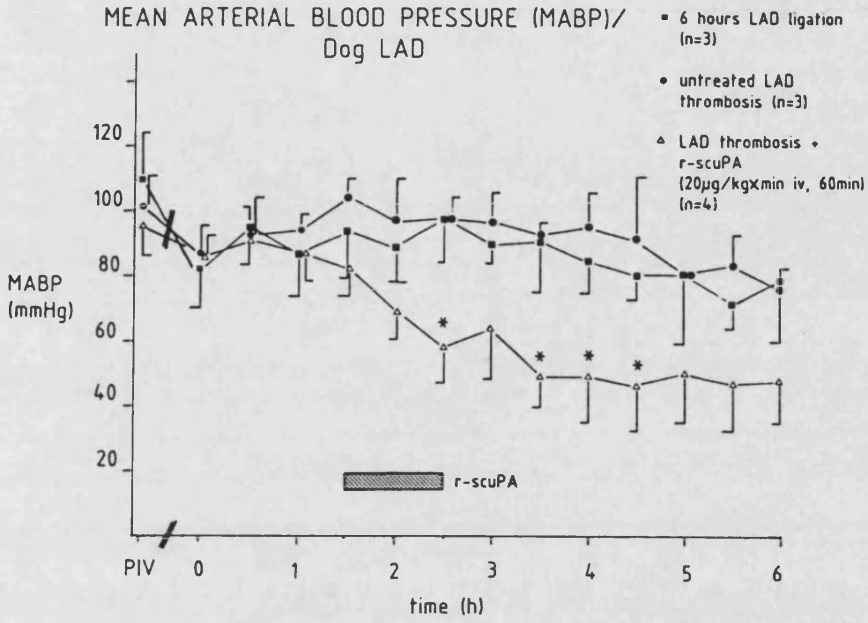


Diagram 15

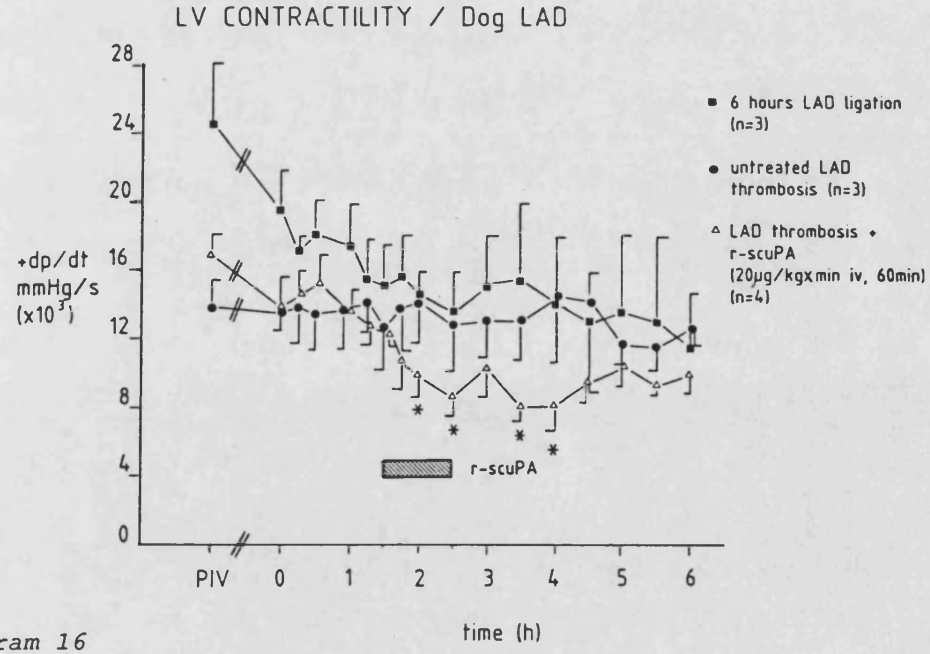


Diagram 16

*<u>Diagrams 15 and 16:</u> Mean arterial blood pressure (MABP) [mm Hg] and LV contractility (+dP/dt) [mm Hg s^{-1}]. Dogs underwent (a) 6 h LAD ligation, (b) 6 h LAD thrombotic occlusion, or (c) r-scu-PA infusion 90 min after LAD thrombosis ($\bar{x} \pm$ S.E.M.). * = $p < 0.05$ r-scu-PA- group (a) versus other 2 groups pooled (b+c), 2-tailed t-test. PIV = preischaemic value.*

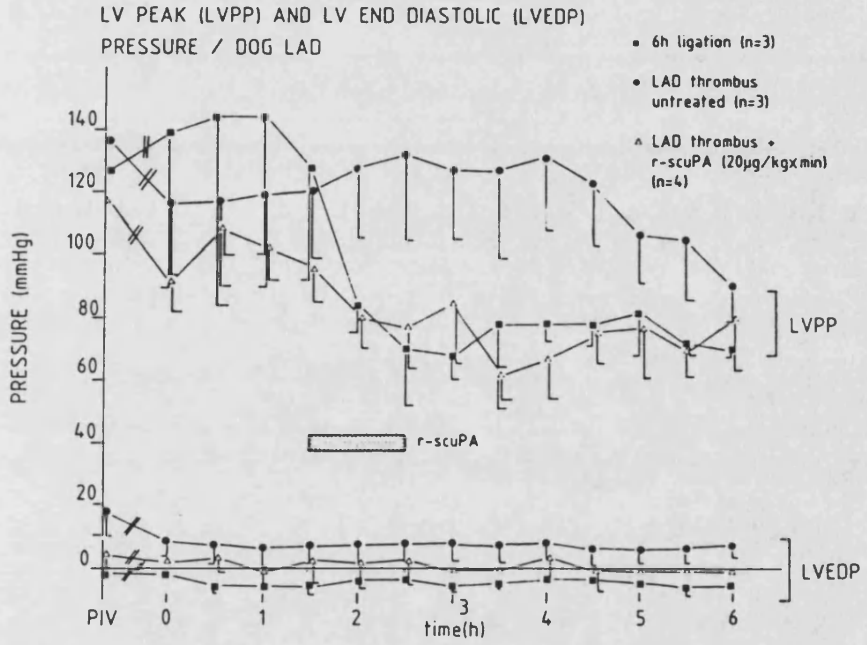


Diagram 17

<u>Diagram 17:</u> Left ventricular peak- and end-diastolic pressure (LVPP/LVEDP) [mm Hg] ($\bar{x}$ ± S.E.M.). Dogs underwent (a) 6 h LAD ligation, (b) 6 h LAD thrombotic occlusion, and (c) r-scu-PA infusion starting 90 min after LAD thrombosis. No significant difference between group (c) and groups (a) + (b) (pooled) was recorded using a 2-tailed t-test. PIV = preischaemic value.

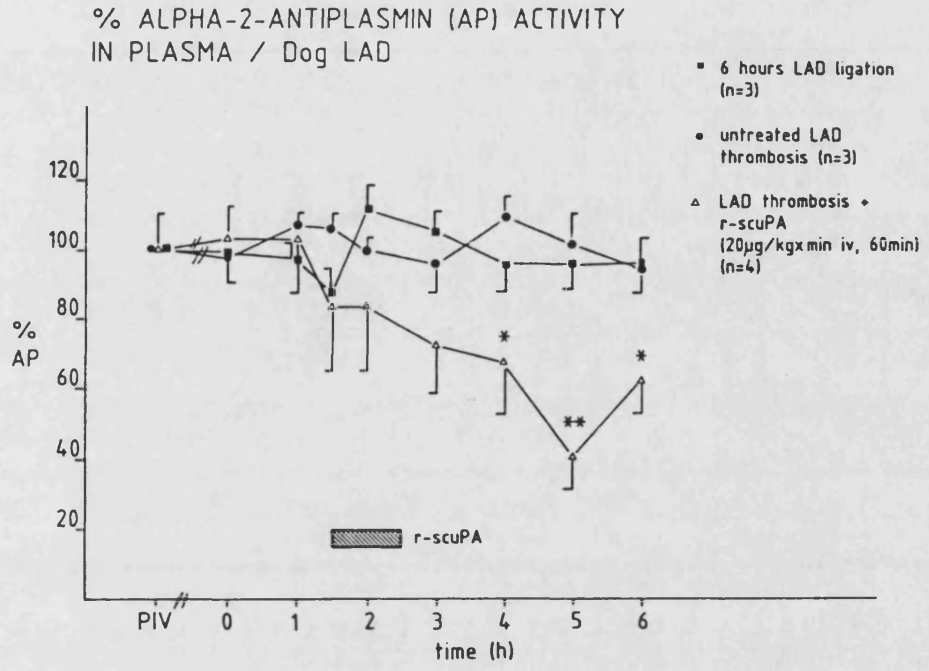


Diagram 18

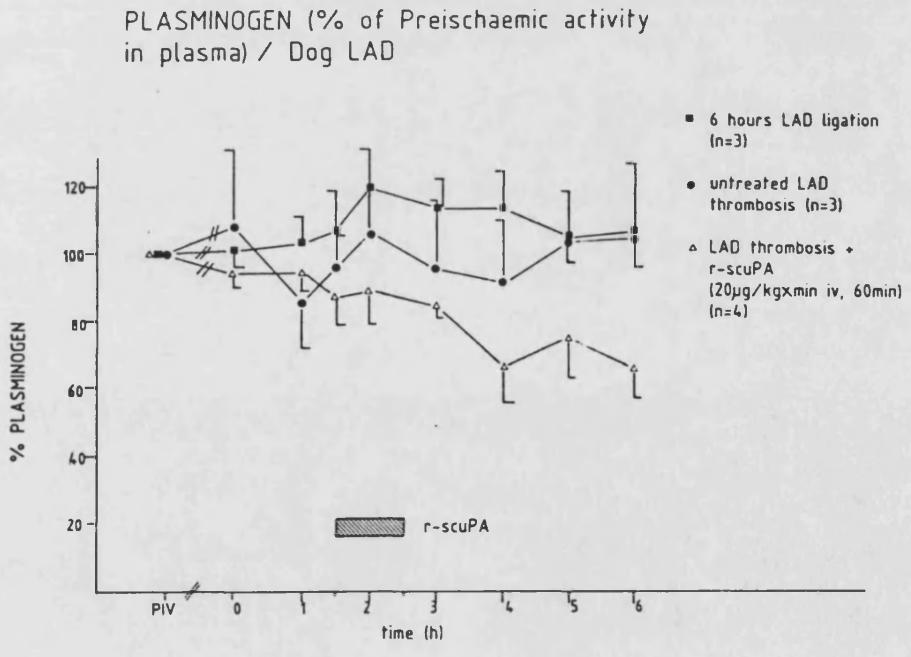


Diagram 19

Diagrams 18 and 19: α_2*-antiplasmin (AP) and plasminogen activities (both % of baseline) (x ± S.E.M.) in plasma of dogs undergoing LAD a) ligation, b) thrombosis or c) thrombosis followed by treatement with r-scu-PA.*
** = $p < 0.05$; ** $p < 0.01$, 2-tailed t-test, r-scu-PA group (c) versus other 2 groups (a+b) pooled. PIV = preischaemic value.*

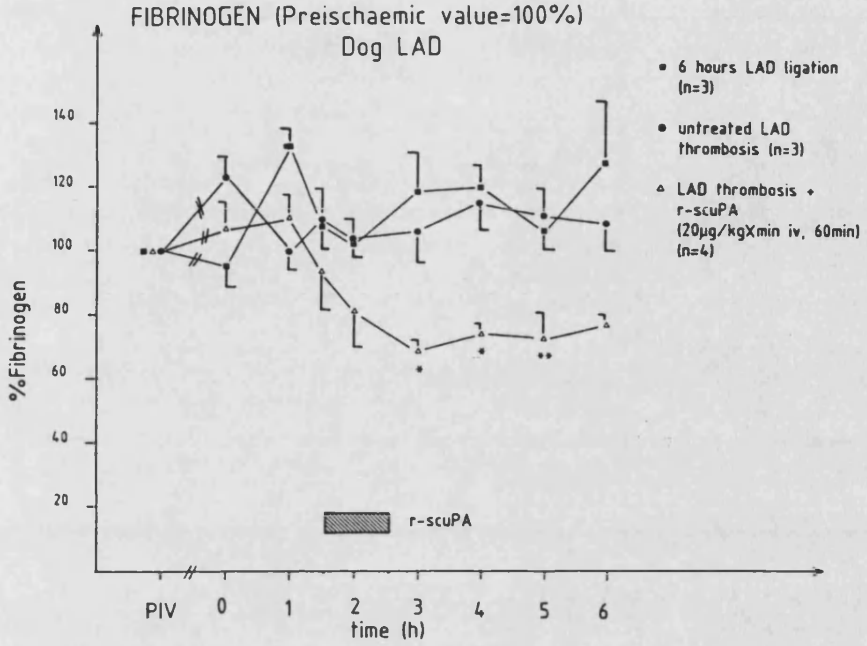


Diagram 20

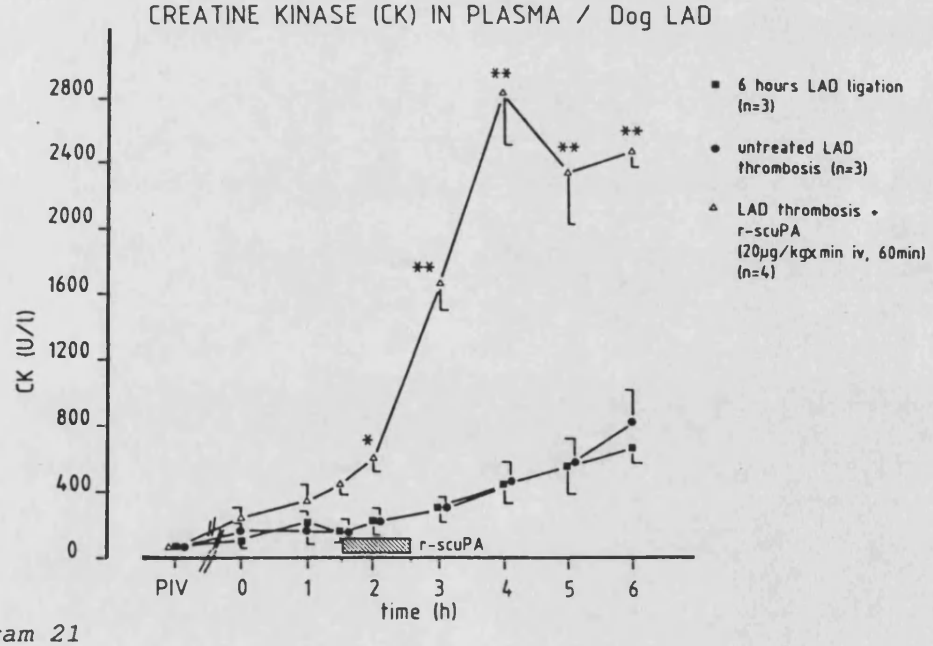


Diagram 21

Diagrams 20 and 21: Fibrinogen [% of pretreatment concentration] and creatine kinase activity (CK) [U/l] in plasma ($\bar{x} \pm$ S.E.M.). Dogs underwent either a) 6 h LAD ligation, b) LAD thrombosis or c) LAD thrombosis followed by r-scu-PA infusion.
** = $p<0.05$, ** = $p<0.01$, 2-tailed t-test, unpaired data, r-scu-PA group (c) versus pool of other 2 groups (a+b).*
PIV=preischaemic value.

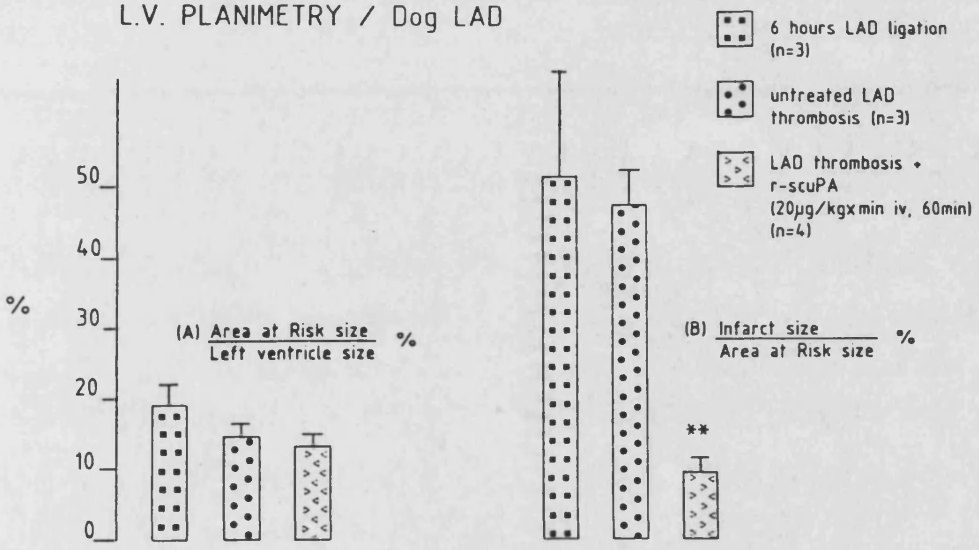


Diagram 22

Diagram 22: Left ventricular planimetry showing: (A) the anatomic area at risk as % of the total left ventricular myocardium (AaR/LV) and, (B) the infarct size as % of the area at risk (I/AaR) ($\bar{x} \pm$ S.E.M.). Infarct sizes were significantly smaller after r-scuPA treatment ($p<0.01$, 2-tailed t-test unpaired data, versus pool of other 2 groups.

HAEMODYNAMIC VARIABLES / Dog Infarct LAD

			Time [h]																
	group	n	PIV	0	0.25	0.5	1	1.25	1.5	1.75	2	2.5	3	3.5	4	4.5	5	5.5	6
HR	a+b	6	177 (10)	201 (4)	201 (10)	204 (8)	204 (8)	204 (8)	204 (9)	195 (11)	198 (7)	198 (9)	201 (8)	207 (10)	213 (10)	216 (8)	213 (7)	219 (7)	213 (8)
	c	4	172 (15)	189 (4)	184 (4)	176* (4)	175* (4)	178* (6)	178 (6)	177 (7)	178 (7)	174 (3)	178* (5)	196 (8)	185 (6)	197 (8)	194 (6)	192* (7)	190 (7)
MABP	a+b	6	106 (6)	84 (4)	92 (5)	93 (5)	91 (7)	- -	96 (7)	100 (7)	90 (7)	97 (8)	94 (5)	92 (9)	92 (11)	88 (11)	81 (12)	79 (12)	77 (12)
	c	4	96 (9)	87 (6)	89 (6)	91 (8)	87 (9)	- -	82 (8)	87 (6)	60 (8)	58* (11)	64 (16)	49* (9)	49* (14)	46* (14)	50 (15)	47 (14)	48 (13)
LV+dP/dt	a+b	6	1816 (249)	1597 (202)	1517 (150)	1521 (171)	1527 (171)	1460 (108)	1449 (159)	1435 (154)	1449 (150)	1433 (148)	1356 (181)	1415 (199)	1415 (199)	1361 (180)	1211 (164)	1211 (178)	1232 (130)
	c	4	1675 (249)	1375 (131)	1425 (155)	1500 (168)	1350 (132)	1263 (85)	1200 (71)	1075 (131)	998* (92)	850* (104)	1025 (165)	800* (71)	800* (122)	938 (99)	1063 (138)	913 (59)	975 (111)
LVPP	a+b	6	133 (15)	125 (22)	131 (26)	128 (26)	129 (25)	125 (17)	124 (17)	110 (16)	110 (22)	107 (22)	103 (21)	107 (20)	110 (18)	105 (16)	97 (11)	92 (13)	83 (7)
	c	4	117 (7)	92 (7)	97 (11)	108 (12)	102 (10)	99 (12)	95 (10)	83 (11)	79 (10)	76 (13)	85 (20)	62 (8)	67 (13)	76 (11)	77 (16)	69 (8)	80 (17)
LVEDP	a+b	6	6 (3)	6 (3)	7 (3)	8 (2)	8 (2)	6 (2)	8 (2)	7 (2)	7 (2)	6 (3)	7 (3)	8 (2)	6 (3)	7 (2)	7 (2)	8 (2)	6 (2)
	c	4	9 (2)	10 (3)	11 (1)	9 (2)	9 (2)	11 (3)	12 (3)	12 (3)	11 (3)	12 (4)	9 (4)	9 (4)	11 (3)	9 (4)	9 (4)	10 (4)	9 (4)

Table 1: **Haemodynamic variables / Dog LAD**

Group a dogs, 6 h LAD ligation (n=3); group b, 6 h stable LAD thombotic occlusion (n=3), therefore both groups (a+b) pooled. Group c: r-scu-PA infusion (from t=1.5 h to t=2.5 h) after LAD thrombosis (n=4). HR = heart rate (beats/min); MABP = mean arterial blood pressure (mm Hg), LV +dP/dt = maximum rate of increase in left ventricular (L.V.) pressure (mm Hg/sec); LVPP = L.V. peak pressure (mm Hg); LVEDP = L.V. end diastolic pressure (mm Hg); PIV = preischaemic value. All values $\bar{x} \pm$ S.E.M.. * = $p < 0.05$, Student's t-test, 2 tail, unpaired data, group c versus groups a + b combined.

PLASMA ASSAYS / Dog Infarct LAD (table 2)

	group	n	Time [h] PIV	0	1	1.5	2	3	4	5	6
AP	a+b	6	100	100 (1)	105 (3)	102 (5)	103 (7)	99 (5)	107 (5)	101 (7)	96 (6)
	c	4	100	103 (11)	103 (10)	85 (19)	84 (19)	72 (14)	68 * (15)	50 ** (10)	63 * (10)
PLG	a+b	6	100	107 (16)	90 (10)	99 (8)	110 (18)	100 (16)	97 (15)	103 (11)	106 (16)
	c	4	100	94 (4)	94 (5)	87 (8)	89 (10)	84 (4)	66 (11)	75 (12)	65 (8)
FIB	a+b	6	100	127 (8)	120 (2)	108 (7)	103 (4)	113 (12)	118 (11)	108 (6)	124 (19)
	c	4	100	107 (8)	110 (7)	94 (12)	81 (11)	69 * (3)	74 * (3)	72 ** (8)	77 (1)
CK	a+b	6	65 (7)	138 (32)	193 (33)	161 (31)	224 (33)	307 (43)	445 (62)	575 (85)	759 (113)
	c	4	62 (12)	235 (55)	373 (73)	446 (81)	607 * (160)	1647 ** (350)	2665 ** (183)	2357 ** (82)	2493 ** (68)

INFARCT SIZE / Dog LAD (table 3)

	AaR/LV (%)	I/AaR (%)
6 h LAD ligation (group a)	23.8 15.6 17.2	24.5 74.4 56.3
(x ± S.E.M.)	18.9 ± 2.5	51.7 ± 14.6
6 h LAD thrombosis (group b)	11.7 15.7 16.5	52.1 38.5 52.3
(x ± S.E.M.)	14.6 ± 1.5	47.6 ± 4.6
(a + b)	(16.8 ± 1.6)	(49.7 ± 6.9)
LAD thrombosis + r-scu-PA (group c)	7.9 18.8 13.7 12.5	12.7 11.3 7.0 6.3
(x ± S.E.M.)	13.2 ± 2.2	9.3 ± 1.6 **

tables 2 and 3: PLASMA ASSAYS and INFARCT SIZE / Dog Infarct LAD
Dogs were subjected to 6 h LAD ligation (group a), or stable thrombotic occlusion (group b), (groups a + b pooled). r-scu-PA was infused from t = 1.5 h to t = 2.5 h following LAD thrombosis (group c).
table 2: AP= α-2-antiplasmin activity; PLG= plasminogen activity (both as % of preischaemic value (PIV)), FIB= % of baseline concentration for each animal (% of PIV). CK= creatine kinase activity (U/l).
table 3: Individual dog infarct size variables, as % of risk area (I/AaR), and proportion of left ventricle "at risk" (AaR/LV).
All values x ± S.E.M.. * = $p<0.05$; ** = $p<0.01$ versus groups a and b combined, 2-tailed t-test, unpaired data

3.2 Results/Dog LCX thrombolysis experiments r-scu-PA taprostene

A total of 30 dogs were used in this study, 5 of which were excluded for the following reasons: Ventricular fibrillation prior to infusion (n =1); coronary vein or artery ruptured during preparation (n =2); no change from normal ECG following ligation (n = 1); abnormal ECG prior to ligation (n = 1). All of the remaining 25 dogs fulfilled the following inclusion criteria: 6 hour survival following initial thrombosis; ECG S-T segment change (≥ 0.2 mV elevation starting within 5 minutes of the initial LCX occlusion) accompanied by a visible cyanosis of the ischaemic area, and stable thrombotic occlusion up to the start of the r-scu-PA (or saline) infusion. The 25 dogs were randomly allocated to the following treatments starting 90 min after thrombotic occlusion of the LCX:

group I vehicle controls (n = 4)

group II r-scu-PA infusion i.v. (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 30 min, n = 9)

group III combined i.v. infusion of r-scu-PA (as group II) plus low dose taprostene (0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 2 h, n = 6)

group IV combined i.v. infusion of r-scu-PA (as group II) plus high dose taprostene (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 2 h, n = 6)

(diagram 11)

Preocclusion LCX-flow [ml/min] (mean ± S.E.M.) was 19.3 ± 1.3, 19.0 ± 1.0, 18.9 ± 1.5 and 18.7 ± 1.3 for groups 1 - 4 with no statistical difference between the groups.

3.2.1 LCX artery patency/Dog

Spontaneous recanalisation occurred in none of the 4 control animals (group I). r-scu-PA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.) for 30 minutes led to successful recanalisation in all animals tested (groups II, III, IV, n = 21). Concomitant infusion of taprostene (0.10 $\mu g \cdot kg^{-1} \cdot min^{-1}$: group III and 0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$: group IV) slightly reduced the mean recanalisation time (time from start of infusions to the onset of reperfusion as detected by the flow meter: > 1.0 $ml \cdot min^{-1}$) from 22.0 ± 2.1 minutes (group II) to 18.2 ± 1.7 minutes and 20.2 ± 2.0 minutes in groups III and IV respectively. This slight reduction in mean lysis time by taprostene was not significant however. The duration of initial LCX occlusion was therefore approximately 110 min in all three r-scu-PA treated groups (groups II, III and IV), as the infusion of r-scu-PA was started 100 min after LCX occlusion (table 7).

3.2.2 Haemodynamic parameters/Dog LCX/Results

Mean arterial blood pressure (MABP)/LCX/Dog

MABP values preceding LCX thrombotic occlusion were similar for all groups (range of group means before ischaemia 135 ± 11 mm Hg to 183 ± 5 mm Hg ($\bar{x}$ ± S.E.M.); individual range: 181 mm Hg to 107 mm Hg). Within minutes of LCX occlusion in all groups a ≈ 20 % fall in MABP was seen, followed by a slight recovery up to 100 minutes when the infusions were started. In the 2 groups receiving taprostene and r-scu-PA (III and IV) a trend to slightly lower MABP during and after taprostene infusion was observed when compared with both the taprostene untreated groups (I and II). The relative MABP decrease associated with taprostene infusion was slight (≈ 20 mm Hg difference between corresponding group means) and prolonged but not significant (diagram 24, table 4).

In contrast to the LAD experiments, the LCX thrombus animals showed virtually no change in MABP as a result of r-scu-PA infusion, when compared with vehicle infusion.

Left ventricular peak (systolic) (LVPP) pressure/LCX/Dog

The peak ventricular pressure decreased shortly after LCX occlusion in all groups from a similar mean preischaemic value (group range of means: 137 ± 10 mm Hg to 152 ± 8 mm Hg ($\bar{x}$ ± S.E.M.), individual range: 104 - 172 mm Hg). The LVPP continued to decrease over the 6 hour experimental period in all groups despite restoration of LCX patency in groups II to IV. A noticeably more rapid decline in LVPP took place during the infusion of the higher dose (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$) of taprostene (group IV). In spite of this, no significantly different LVPP value was observed between any 2 of the 4 treatment groups at equivalent time points (diagram 23, table 4).

L.V. contractility (+dP/dt)/LCX/Dog

Variations in the group means of the left ventricular +dP/dt (maximum rate of increase in ventricular pressure) prior to ischaemia were large (range 1000 - 2400 mm Hg/s) and at this point 2 group means were statistically different ($p < 0.05$, t-test; group I: 2050 ± 132 ($\bar{x}$ ± S.E.M.) and group IV: 1400 ± 121 mm Hg/s). The groups mean values tended to decrease in a convergent manner, however, minutes after LCX occlusion by about 25 % to a common value. Regardless of the different treatments, and of the restoration of LCX patency in all members of groups II to IV, the left ventricular contractility decreased slowly and uniformly over the 6 hours in all 4 groups, so that the 6 hour contractility values were 38 ± 7%, 63 ± 6 %, 53 ± 5 % and 51 ± 6 % (mean % of preischaemic value

±S.E.M.) for treatment groups I to IV respectively. Thus although the absolute mean contractility was lowest in the high dose taprostene group, and significantly different to the non-reperfused controls (group I, $p < 0.05$, t-test, 2-tail) at this time, the maximum % decrease in contractility occurred in the untreated control group. This may be an artefact related to the excessively high contractility in this group (group I) prior to ischaemia (diagram 25, table 5).

Heart rate (HR) and Double product (HR x MABP)/LCX/Dog

The heart rates remained virtually unchanged from the preischaemic values (≈ 200 beats/min, range: 165 - 225 /min) in all groups over the 6 hour period regardless of the different treatments.
As a result, the double product (HR x MABP) - an index of myocardial oxygen demand - followed a similar course to the blood pressure (MABP) over the experimental period. In brief, no significant changes between treatment groups were observed although in taprostene treated groups (groups III and IV) a marginally lower energy demand may have resulted from slightly lower MABP values.
This very slight effect was at no time significant when compared with either continuous occlusion or r-scu-PA alone treated groups (diagram 27, table 6).

Arrhythmias/LCX/Dog

No arrhythmic activity was observed before LCX thrombosis. Following thrombosis a low mean frequency of ectopic beats was observed in all groups (1 - 2 per 20 beats). A dramatic increase in arrhythmic activity (approximately 4 x) occurred in all animals treated with fibrinolytic agent and was temporally associated with the onset of reperfusion as detected by the flow probe. In taprostene-cotreated groups (III and IV) dose-dependent reduction in reperfusion arrhythmias was observed when compared with the r-scu-PA-alone group (I) (diagram 29, table 8). This was significant for the higher dose of taprostene over the 3 to 6 hour period ($p < 0.05$ versus group II, 2-tailed t-test, unpaired data). The relatively low incidence of arrhythmia in the continuous occlusion group (gp I) tended to increase towards the end of the experimental period (diagram 29, table 8).

3.2.3 Infarct size parameters/Dog LCX/Results

LV myocardium "at risk"

The proportion of LV myocardium directly supplied by the LCX (% AaR/LV) was found, with the balanced pressure ex vivo dye perfusion method, to be relatively constant for all animals (38.8 1.1 %; n=25, range: 26.6 % to 47.7 %). The mean group values were very consistent (see diagram 28, table 7).

group I: 42.4 ± 3.7 %

II: 38.4 ± 1.6 %

III: 39.3 ± 2.5 %

IV: 36.7 ± 2.4 %

Infarct size

Expressed as a % of the area at risk (% I/AaR) the relative infarct sizes (T.T.C. unstained) at 6 hours were found to be:

group I: 48.3 ± 7.7 %

II: 25.3 ± 3.7 % ($p < 0.05$ vs group I)

III: 21.3 ± 6.5 % ($p < 0.05$ vs group I)

IV: 17.1 ± 3.5 % ($p < 0.01$ vs group I)

Thus r-scu-PA treatment was associated with a reduction in relative infarct size compared with the control animals (group I) in which LCX recanalisation was not achieved. Taprostene cotreatment showed a dose-related trend to further decrease the proportion of myocardium staining as necrotic at 6 hours (diagram 28, table 7). This possible further reduction in acute infarct size by taprostene was not statistically significant however when compared to the r-scu-PA-alone treated group. The taprostene/r-scu-PA combination treatment groups (groups III and IV) exhibited, like the r-scu-PA alone group (group II), statistically smaller infarcts than the untreated group (group I). The restoration of LCX artery patency after 112 to 127 min (group II) was associated with a major (48 %) reduction in infarct size. Taprostene under the conditions tested, starting late in the ischaemia phase, had a minor effect, if any, on the TTC stained morphometric acute infarct size, beyond that of r-scuPA alone. The magnitude of this additional effect however would require larger numbers per treatment group to be employed in order to demonstrate statistical significance.

3.3.2 Haemostatic parameters/LCX Dog/Results

Plasminogen/LCX/Dog

All dogs, by definition, had a plasma plasminogen level of 100 % after surgical preparation and prior to ischaemia and infusion(s). The r-scu-PA untreated dogs (group I) retained their baseline plasminogen levels over the 6 hours of thrombotic occlusion of the LCX (range of minima: 91 - 95 %). In the other groups, which were all treated with r-scu-PA, an initial plasminogen nadir was observed between 30 minutes and 1 hour after completing the r-scu-PA infusion (range of minima for each group: group II: 61 - 105 %, group III: 63 - 88 %, group IV: 56 - 96 %). Taprostene coadministration, at the lower dose, with r-scu-PA singificantly ($p < 0.02$, t-test, 2-tail) lowered the plasminogen levels compared with r-scu-PA untreated controls (group I) although the r-scu-PA alone treated group (group II) failed to differ significantly from the untreated group (group I). The high dose taprostene plus r-scu-PA group (group IV) showed marginally lesser depression of relative plasminogen levels than the low dose taprostene plus r-scu-PA group (group III) and again a significant depression of plasminogen compared to controls (group I) (diagram 31, table 9).

α_2-Antiplasmin (AP)/LCX/Dog

Again, by definition, the pretreatment AP activity for each animal was 100 %. In the control group (group I) the activity remained unchanged over the 6 hour period (range of minimum AP activity

observed: 91 - 94 % of baseline). In common with the plasminogen assays, the greatest indirect evidence of plasmin formation was in the low dose taprostene plus r-scu-PA group (group III) ($p < 0.02$, t-test, 2-tail vs. control group I). This was evident at multiple time points after completing the infusion of r-scu-PA. In the r-scu-PA alone treated group (group II) AP activities were significantly reduced at t = 5 h and t = 6 h (range of nadirs for each animal in this group: 52 - 105 % of baseline value) but this was to a lesser extent than in group III. Unexpectedly, high dose taprostene plus r-scu-PA infusions did not cause even greater AP activity depletion than in the low dose taprostene/r-scu-PA group (group III), but the effect of AP activity depletion seemed to have been marginally attenuated implying reduced systemic plasmin generation at the higher taprostene dose level. The range of minimum AP activity observed was from 59 - 88% in group III and from 66 to 98% in group IV (% of baseline AP activity for each animal) (diagram 30, table 9).

Plasma fibrinogen/LCX/Dog

The mean initial values of plasma fibrinogen (mg/100 ml) were 121± 9, 114 ± 5, 136 ± 17 and 125 ± 12 ($\bar{x}$ ± S.E.M.) for groups I to IV, respectively. In individual animals, plasma fibrinogen concentrations varied from 88.5mg/100 ml to one animal with an extremely high level of 313mg/100 ml fibrinogen. This animal can be identified in table 7 as being the group III animal with a peak CK value of 337 U/l. Infusion of r-scu-PA alone, or in combination with taprostene, did not alter the mean plasma fibrinogen levels

significantly when compared with the baseline values of the same group or when corresponding time points were compared between different treatment groups.

The range of minimum plasma fibrinogen concentrations (nadirs, expressed as a percent of the baseline value for each animal) was as follows for each of the groups:

group I : 75 - 98 %

group II : 70 - 89 %

group III: 61 - 84 %

group IV : 70 - 94 %.

The mean plasma fibrinogen concentrations are given in diagram 32, table 9 shows the % fibrinogen (% of each dog's baseline (PIV) value) to enable relative changes in fibrinogen levels between groups to be visualized.

3.2.5 Plasma CK activity/LCX Dog/Results

Plasma CK activity following surgical preparation and immediately before LCX thrombus formation was < 50 U/l for all dogs (individual range 9.5 to 40.0 U/l). In all groups a gradual increase in plasma CK activity was observed during the LCX occlusion period. In the continuous occlusion group (group I) the plasma CK activity increased slowly and linearly over the 6 hour experimental (ischaemic) period, resulting in a group mean value (± S.E.M.) of 766 ± 87 U/l at 6 h (range 557 - 978 U/l). In animals treated with r-scu-PA alone (group II) a rapid rise in CK activity coincided with the onset of reperfusion. This rate of rise was almost constant over the observed period, and a mean CK activity of 1990 ± 242 U/l ($\bar{x}$ ± S.E.M., range: 1220 to 2945 U/l) was recorded at the end of the experiment. High dose taprostene infusion (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$, group IV) reduced the rate of rise of CK activity following r-scu-PA-induced recanalization when compared with group II, resulting in a mean CK activity of 750 ± 123 U/l ($\bar{x}$ ± S.E.M, range: 399 to 1252 U/ml) at the end of the experiment. This was significantly lower than the mean value of plasma CK activity of the r-scu-PA alone treated group (group II) ($p < 0.02$, Student's t-test, 2-tail). The lower dose taprostene infusion in group III showed no significant decrease in CK release compared to group II. A lesser effect of this lower dose of taprostene to attenuate the rise in CK activity may have been masked by extraordinarily high CK activity in one member of the group (6 h value: 7152 U/l, other values in this group ranged from 337 to 1641 U/l at this time) (diagram 33, table 9).

Summary of Dog LCX thrombolysis experiments

To summarize the results of the LCX experiments, r-scuPA infusion (20 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 30 min duration) led to recanalization of the LCX in all animals treated (table 7). Taprostene coadministration with r-scuPA did not markedly affect the rate of recanalization. Taprostene coadministration also did not cause a significant change in any of the following haemodynamic parameters when compared to r-scuPA alone: left ventricular peak pressure, mean arterial blood pressure, heart rate and rate x pressure product (diagrams 23, 24, 26, 27). High dose taprostene cotreatment (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 2 hour infusion) caused a significant decrease in ventricular contractility after completion of the infusions compared to the r-scuPA alone group (table 5, diagram 25). Also in the high dose taprostene group a significant reduction in the frequency of ventricular ectopic beats was recorded (table 8, diagram 29). As in the LAD experiments thrombolytic recanalization reduced the infarct size in these experiment from 48.3 $\pm$ 7.7% (I/AaR) in the 6 h thrombotic occlusion group to 25.3 $\pm$ 3.7% in the r-scuPA alone group (table 7, diagram 28). Taprostene cotreatment starting after 90 minutes of LCX occlusion produced a trend to a further reduction of infarct sizes as measured by left ventricular planimetry and a significant decrease in the maximum plasma CK activity after reperfusion in the higher dose group (group IV) (diagram 33). Evidence of slight systemic plasmin generation was seen in most groups treated with r-scuPA. Low dose but not high dose taprostene may have enhanced this effect as shown by the changes in plasma levels of α_2-antiplasmin and plasminogen (diagram 30 and 31). Fibrinogen

concentrations were not affected by any of the treatments, in contrast to the LAD experiments where a higher total dose of r-scuPA caused significant decrease (diagram 32).

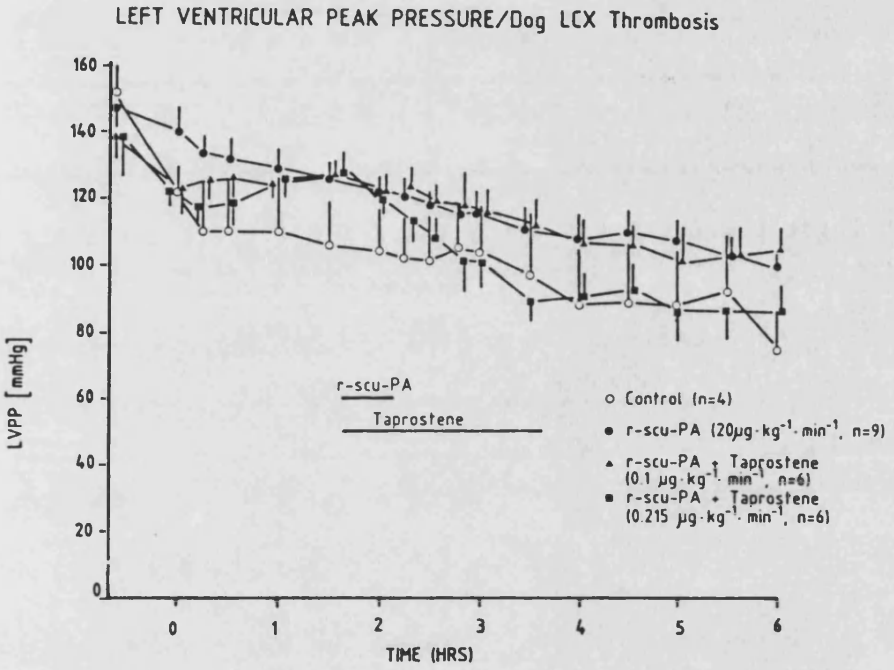


Diagram 23

Diagram 23: Left ventricular peak pressure (LVPP) [mm Hg] ($\bar{x}$ ± S.E.M.). Dogs underwent LCX thrombosis starting at t = 0h followed by: group I vehicle infusion, group II r-scu-PA infusion, groups III and IV r-scu-PA infusion together with low and high dose taprostene infusions respectively. No significant difference ($p>0.05$) was observed (2-tailed t-test, unpaired data).

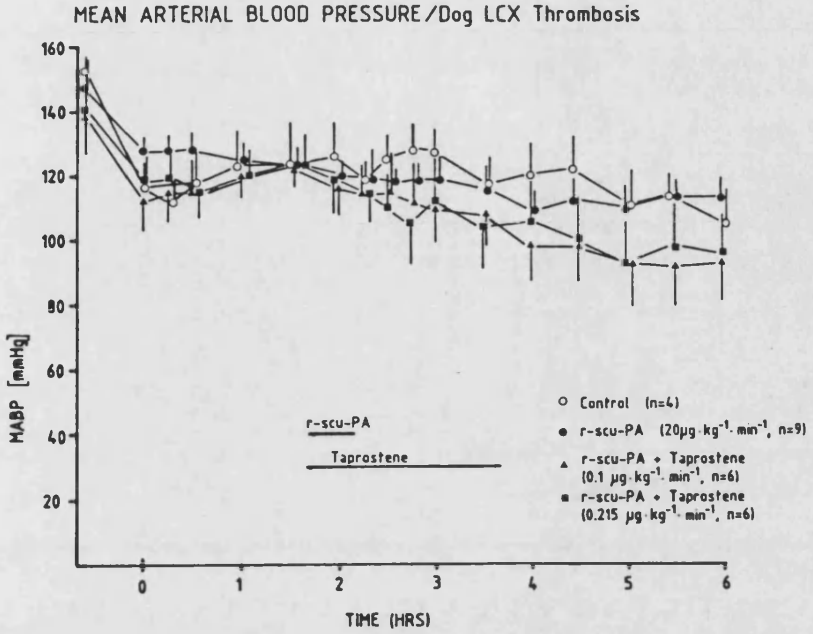


Diagram 24

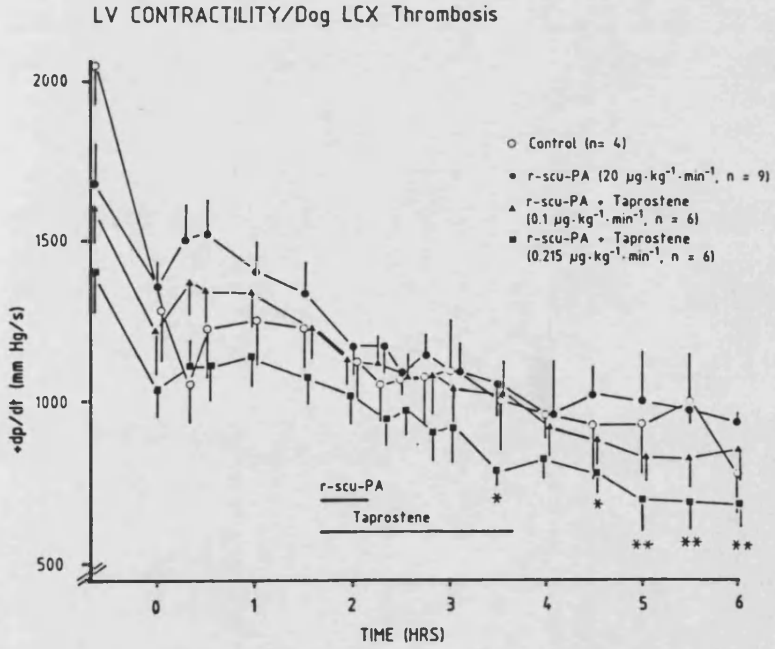


Diagram 25

*<u>Diagrams 24 and 25:</u> Mean arterial blood pressure (MABP) [mm Hg] and left ventricular contractility (+dP/dt) [mm Hg s^{-1}], ($\bar{x} \pm$ S.E.M.). Dogs underwent LCX thrombosis starting at t = 0h followed by: group I vehicle infusion, group II r-scu-PA infusion, groups III and IV r-scu-PA infusion together with low and high dose taprostene infusions respectively. * = p<0.05, ** = p<0.01, 2-tailed t-test, unpaired data, versus control group (LCX thrombosis, vehicle infusions).*

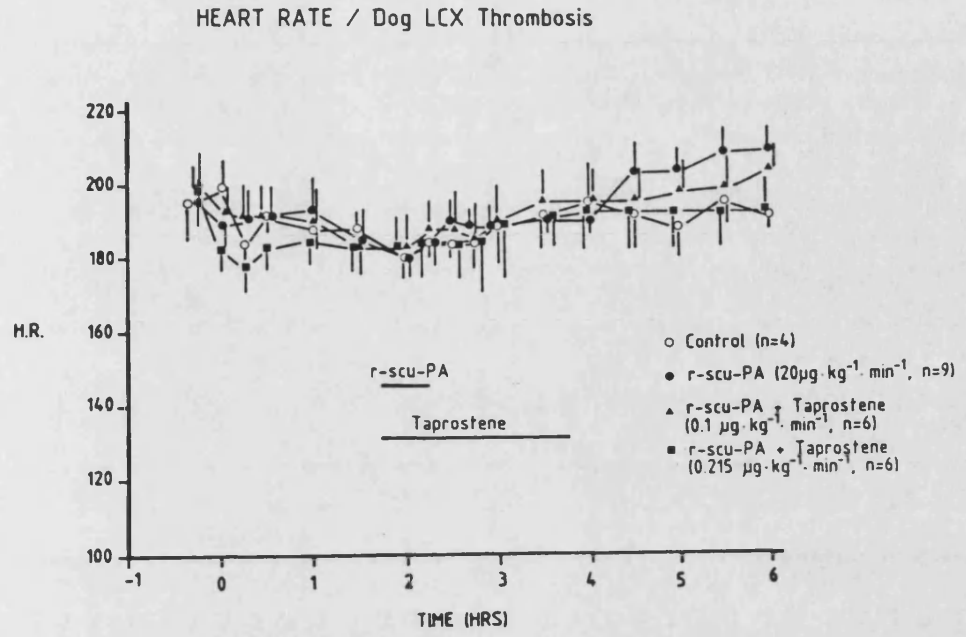


Diagram 26

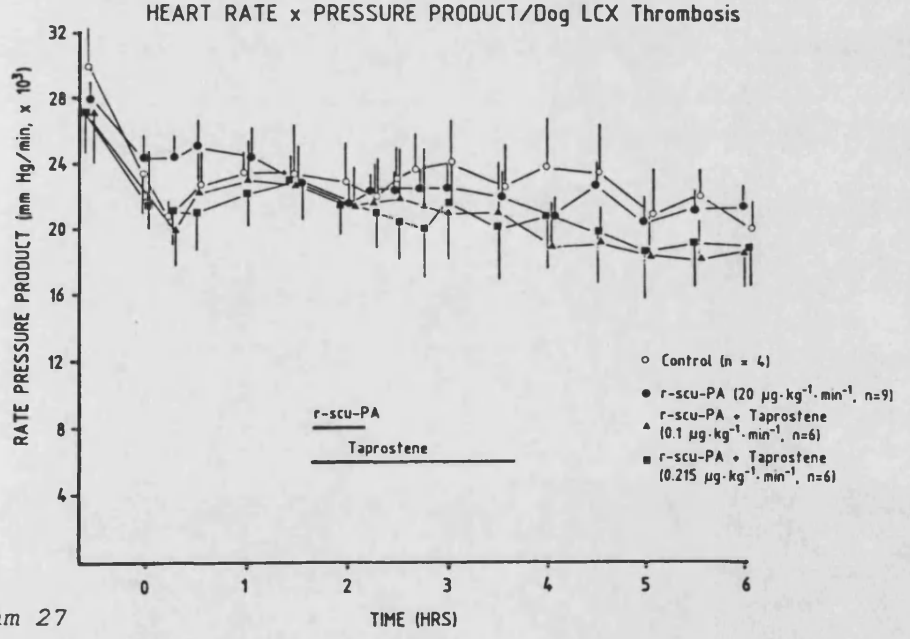


Diagram 27

<u>Diagrams 26 and 27:</u> Heart rate (HR) [beats/min] and heart rate x mean arterial blood pressure [mm Hg/min] ($\bar{x} \pm$ S.E.M.). Dogs underwent LCX thrombosis starting at t = 0h followed by: group I vehicle infusion, group II r-scu-PA infusion, groups III and IV r-scu-PA infusion together with low and high dose taprostene infusions respectively. No significant difference in these 2 variables was found between any 2 groups at corresponding times ($p>0.05$, 2-tailed t-test, unpaired data).

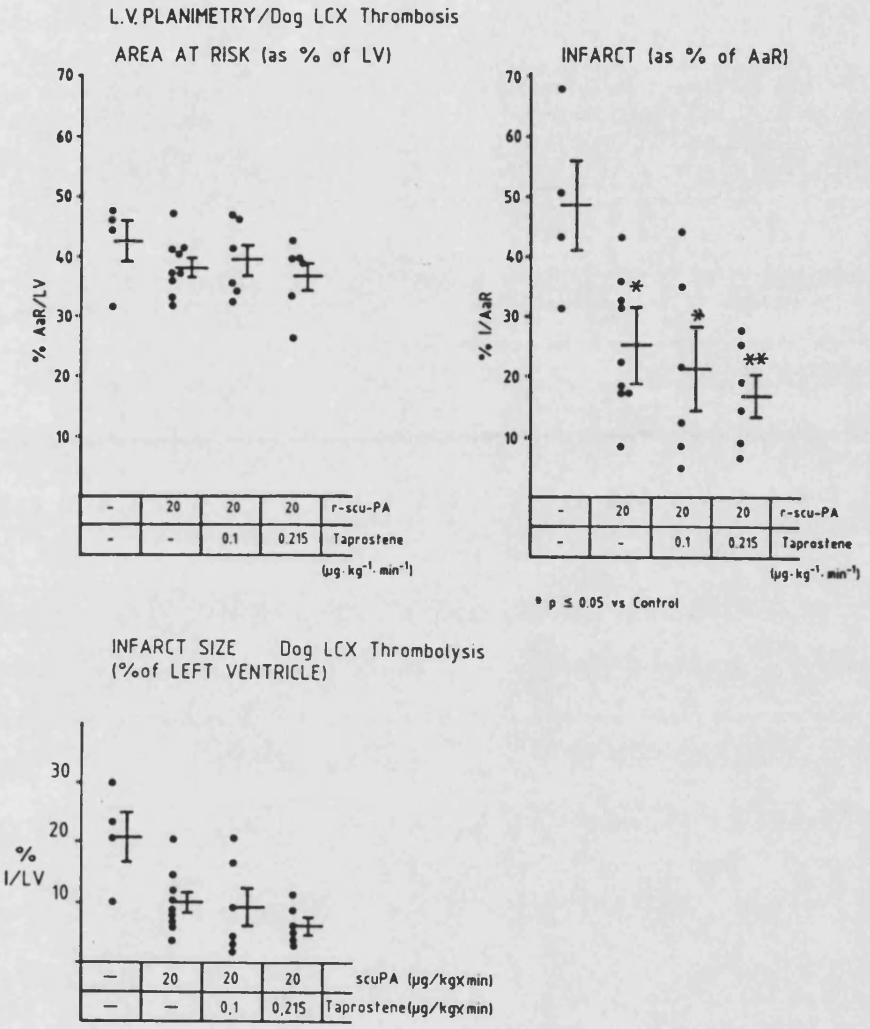


Diagram 28

*Diagram 28: Left ventricular planimetry to measure: (top left) the anatomic area of myocardium "at risk" expressed as % of left ventricle (%AaR/LV); (top right) the infarct size at 6 hours as % of the area at risk (% I/AaR) and (below) - the infarct size as % of left ventricle (% I/LV). Lines represent mean ± S.E.M., individual dogs shown as dots. * = p<0.05, ** p<0.01, 2-tailed t-test, unpaired data versus control group which received no active drug.*

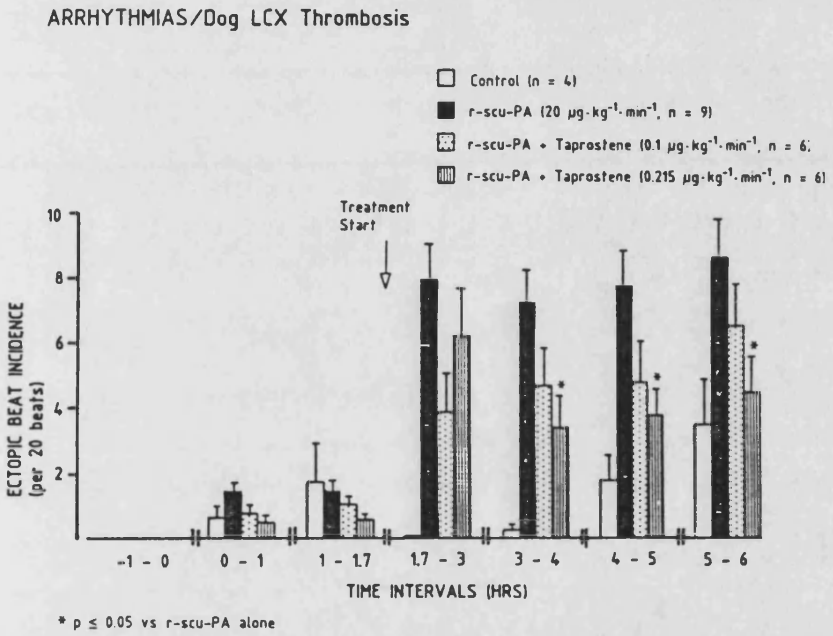


Diagram 29

Diagram 29: Arrhythmic activity [number of ectopic beats per 20 beat sequence] over each of the time intervals defined on the x-axis, ($\bar{x}$ ± S.E.M.). Dogs underwent LCX thrombosis at t = 0h followed by treatment started at t=1.5h of : group I vehicle, II: r-scu_PA (30 min infusion), III and IV r-scu-PA as for II with low (III) and high (IV) dose taprostene infusion (2 h duration).
** = p<0.05, 2-tailed t-test, unpaired data, versus group II (r-scu-PA alone, blacked out columns).*

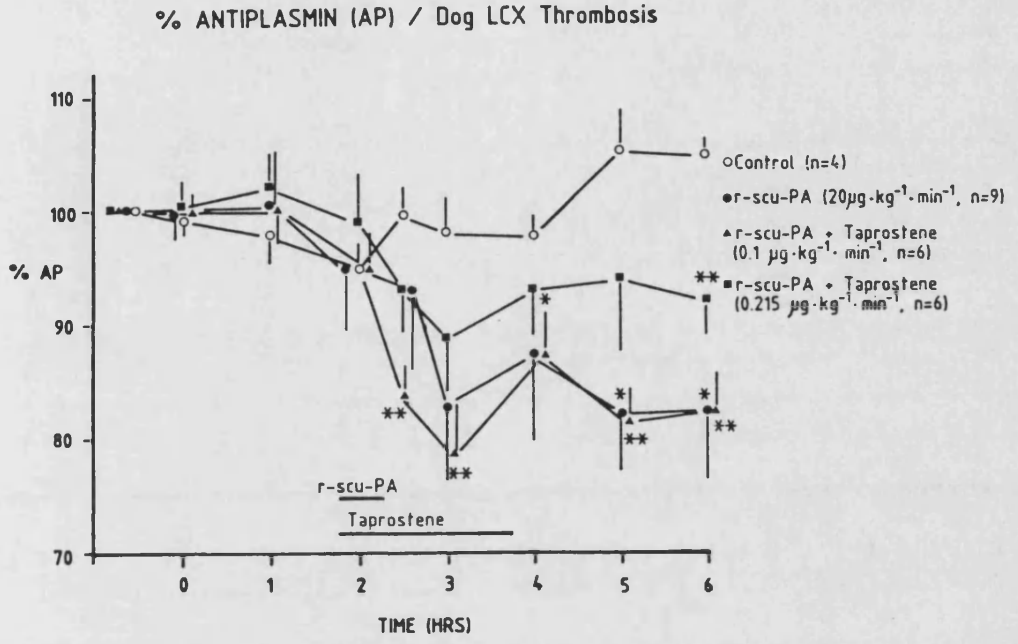


Diagram 30

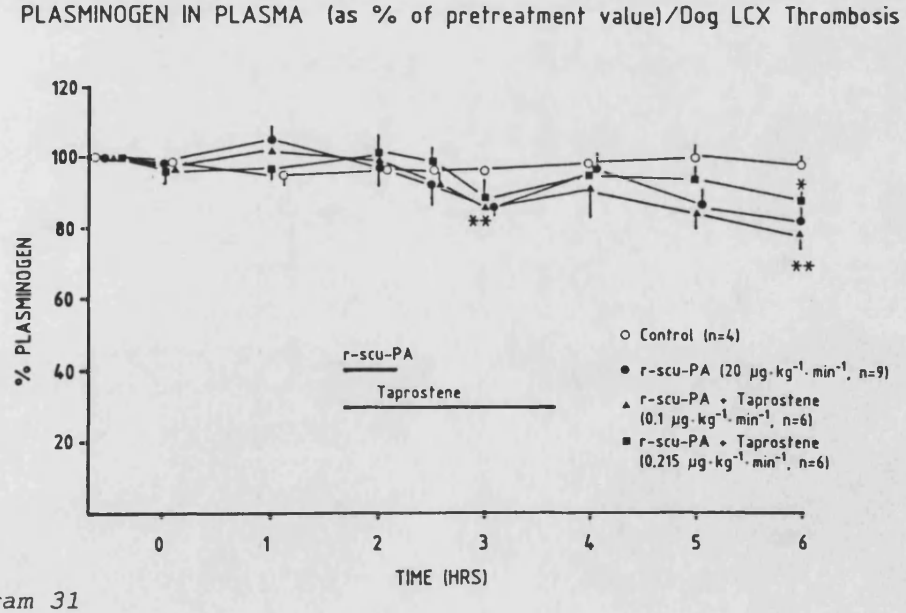


Diagram 31

Diagrams 30 and 31: Plasma α_2-antiplasmin activity (AP) and plasminogen activity (both as % of preischaemic baseline value). Dogs underwent LCX thrombosis starting at t = 0h followed by: group I vehicle infusion, group II r-scu-PA infusion, groups III and IV r-scu-PA infusion together with low and high dose taprostene infusions respectively ($\bar{x} \pm$ S.E.M.).
** = $p<0.05$, ** = $p<0.01$, 2-tailed t-test, unpaired data, versus group I (vehicle controls: hollow circles).*

FIBRINOGEN IN PLASMA (as % of pretreatment value)/Dog LCX Thrombosis

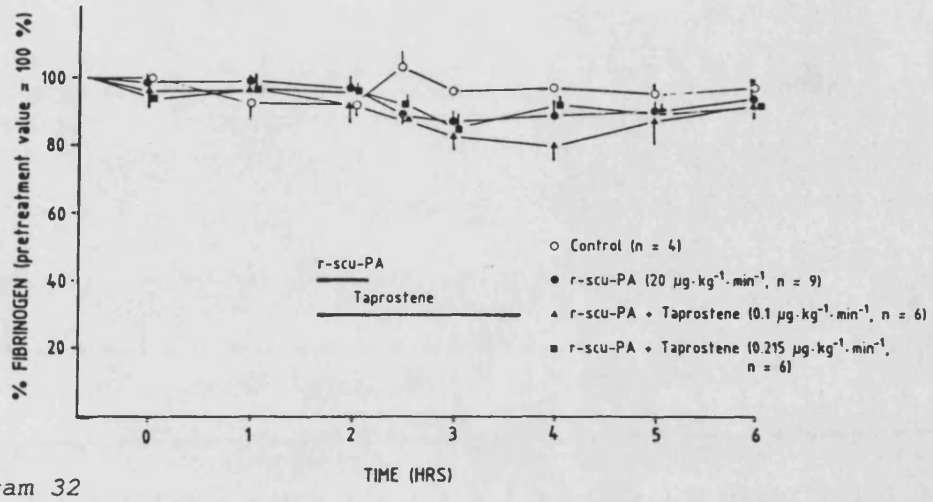


Diagram 32

CREATINE KINASE (CK) IN PLASMA/Dog LCX Thrombosis

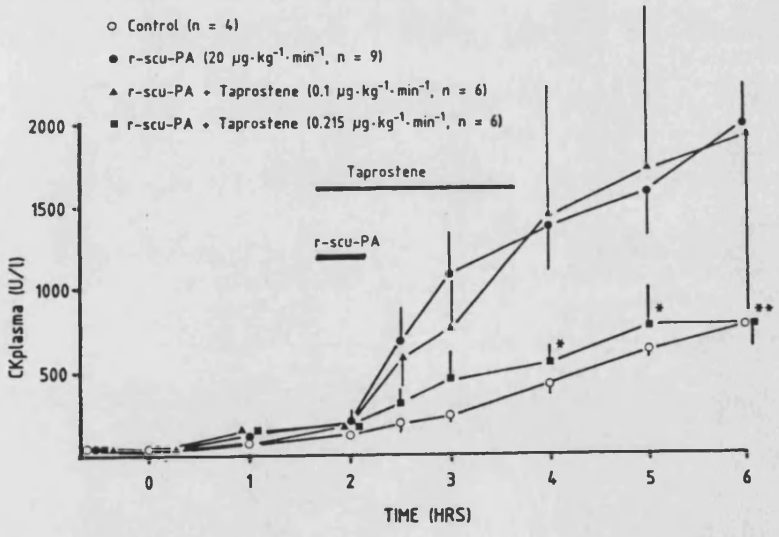


Diagram 33

<u>Diagrams 32 and 33:</u> Plasma fibrinogen concentration [% of preischaemic baseline value] and creatine kinase activity (CK) [U/l], both in plasma. Dogs underwent LCX thrombosis starting at t = 0h followed by: group I vehicle infusion, group II r-scu-PA infusion, groups III and IV r-scu-PA infusion together with low and high dose taprostene infusions respectively ($\bar{x} \pm$ S.E.M.).
** = $p<0.05$, ** = $p < 0.01$, 2-tailed t-test, unpaired data, versus group II (r-scu-PA alone: blacked out circles).*

HAEMODYNAMIC VARIABLES / Dog LCX Thrombosis

group	[µg/kg/min] r-scu- PA	TAP	n	Time [h] PIV	0	0.25	0.5	1	1.5	2	2.25	2.5	2.75	3	3.5	4	4.5	5	5.5	6
LVPP																				
I	0	0	4	152	122	110	110	110	106	104	102	101	105	104	97	88	89	88	93	76
				(8)	(8)	(15)	(16)	(16)	(13)	(13)	(12)	(13)	(14)	(14)	(18)	(17)	(18)	(17)	(17)	(11)
II	20	0	9	148	140	133	132	129	127	122	122	118	115	116	111	108	110	108	103	100
				(7)	(7)	(5)	(6)	(6)	(5)	(5)	(5)	(4)	(3)	(6)	(6)	(7)	(6)	(6)	(6)	(5)
III	20	0.1	6	139	123	125	126	125	127	122	124	120	118	116	112	107	106	101	103	105
				(6)	(8)	(5)	(6)	(4)	(4)	(6)	(6)	(5)	(10)	(7)	(8)	(7)	(6)	(10)	(9)	(7)
IV	20	0.215	6	137	122	117	119	126	127	120	113	108	101	101	89	91	93	87	87	86
				(10)	(4)	(4)	(7)	(5)	(7)	(5)	(6)	(6)	(9)	(7)	(6)	(7)	(8)	(9)	(9)	(8)
MABP																				
I	0	0	4	153	117	112	118	123	123	126	119	125	128	127	117	120	122	110	113	105
				(5)	(9)	(4)	(10)	(11)	(13)	(10)	(9)	(7)	(8)	(7)	(8)	(10)	(10)	(11)	(7)	(11)
II	20	0	9	147	128	128	129	125	123	120	120	118	119	119	116	109	112	100	103	103
				(7)	(4)	(5)	(5)	(5)	(5)	(4)	(5)	(4)	(5)	(7)	(6)	(6)	(7)	(7)	(6)	(6)
III	20	0.1	6	135	112	115	115	120	123	116	114	115	112	110	108	97	98	94	92	93
				(11)	(9)	(9)	(7)	(7)	(7)	(8)	(9)	(10)	(12)	(11)	(13)	(11)	(11)	(13)	(12)	(12)
IV	20	0.215	6	138	118	119	114	120	124	119	114	110	105	112	104	106	100	93	98	96
				(11)	(6)	(9)	(11)	(8)	(9)	(9)	(9)	(10)	(13)	(14)	(14)	(13)	(13)	(13)	(13)	(12)

table 4: HAEMODYNAMIC VARIABLES / LVPP and MABP / Dog LCX
Dogs were subjected to 6 h LCX thrombotic occlusion (group I), or infusions, starting 1.5 h after LCX thrombosis, of r-scu-PA (30 min duration: groups II, III and IV) together with taprostene (TAP) infusion (t = 1.5 h to t = 3.5 h) at low dose (group III), high dose (group IV) or taprostene vehicle (group II). LVPP = left ventricular peak pressure (mm Hg); MABP = mean arterial blood pressure (mm Hg); PIV = preischaemic value.
All values arithmetic mean ± standard error ($\bar{x}$ ± S.E.M.). No intergroup difference ($p < 0.05$, Student's t-test, 2-tail) was observed in either variable.

VENTRICULAR CONTRACTILITY / Dog Infarct LCX

	[µg/kg/min]			+dP/dt [mm Hg/s]																
				Time [h]																
gp	r-scu-PA	taprostene	n	PIV	0	0.25	0.5	1	1.5	2	2.25	2.5	2.75	3	3.5	4	4.5	5	5.5	6
I	0	0	4	2050 (132)	1275 (165)	1050 (119)	1225 (165)	1250 (144)	1225 (125)	1125 (131)	1050 (126)	1075 (144)	1075 (149)	1100 (147)	1000 (163)	950 (166)	925 (175)	925 (155)	1000 (147)	775 (131)
II	20	0	9	1567 (121)	1356 (84)	1500 (115)	1522 (115)	1400 (96)	1333 (88)	1167 (88)	1178 (82)	1089 (65)	1144 (71)	1089 (101)	1050 (93)	956 (77)	1017 (78)	1000 (55)	967 (41)	933 (29)
III	20	0.1	6	1600 (115)	1133 (102)	1367 (99)	1342 (100)	1333 (109)	1217 (95)	1133 (88)	1108 (95)	1067 (76)	1067 (131)	1033 (112)	1017 (111)	917 (95)	883 (79)	825 (73)	825 (96)	850 (92)
IV	20	0.215	6	1400 ♦ (121)	1033 (84)	1100 (86)	1100 (106)	1133 (95)	1067 (71)	1017 (91)	942 (84)	967 (76)	900 (93)	917 (114)	783 ♦ (40)	817 (60)	783 ♦ (60)	700♦♦ (97)	683♦♦ (70)	683♦♦ (70)

Table 5: VENTRICULAR CONTRACTILITY / Dog Infarct LCX
Mean maximum rate of increase in ventricular pressure (+dP/dt) in dogs undergoing left circumflex coronary artery (LCX) thrombosis.
Dogs were grouped (I - IV) according to drug treatment above: taprostene was infused for 2 hours starting at t = 1.5 h at 2 dose levels (groups III and IV) or substituted with placebo (group II). r-scu-PA was infused in groups II, III and IV for 30 min starting at t = 1.5 h.
PIV = preischaemic baseline value.
$\bar{x}$ ± S.E.M. ♦ = $p<0.05$; ♦♦ = $p<0.02$ vs. group I ; Student's t-test, 2-tail, unpaired data.

HAEMODYNAMIC VARIABLES / Dog LCX Thrombosis

	gp	[µg/kg/min] r-scu-PA	TAP	n	Time [h] PIV	0	0.25	0.5	1	1.5	2	2.25	2.5	2.75	3	3.5	4	4.5	5	5.5	6
HR	I	0	0	4	195 (11)	199 (7)	184 (4)	191 (4)	188 (4)	188 (4)	180 (6)	184 (4)	184 (4)	184 (7)	188 (10)	191 (9)	195 (11)	191 (9)	188 (8)	195 (6)	191 (4)
	II	20	0	9	193 (7)	188 (7)	190 (9)	192 (9)	193 (8)	185 (9)	180 (6)	185 (6)	190 (6)	188 (5)	188 (7)	190 (5)	190 (4)	203 (8)	203 (6)	208 (7)	209 (7)
	III	20	0.1	6	200 (9)	193 (8)	190 (10)	193 (9)	190 (9)	183 (8)	183 (9)	188 (8)	188 (11)	185 (11)	190 (10)	195 (9)	195 (9)	195 (9)	198 (9)	198 (9)	203 (7)
	IV	20	0.215	6	198 (7)	183 (6)	178 (7)	183 (6)	185 (7)	183 (9)	183 (9)	183 (9)	185 (10)	185 (13)	188 (13)	190 (9)	193 (11)	193 (11)	193 (11)	193 (10)	193 (10)
MABPxHR																					
	I	0	0	4	30.0 (2.5)	23.4 (2.5)	20.6 (1.1)	22.7 (1.9)	23.3 (1.2)	23.3 (2.9)	22.9 (2.4)	22.0 (2.2)	23.1 (1.8)	23.7 (2.1)	24.1 (2.6)	22.7 (2.5)	23.8 (3.1)	23.5 (3.0)	20.9 (2.8)	22.0 (1.8)	19.9 (1.6)
	II	20	0	9	28.1 (1.0)	24.3 (1.4)	24.3 (1.4)	25.0 (1.8)	24.4 (1.8)	22.9 (1.7)	21.6 (1.1)	22.3 (1.3)	22.4 (1.1)	22.4 (1.2)	22.6 (1.6)	22.0 (1.4)	20.8 (1.2)	22.9 (1.6)	20.4 (1.6)	21.3 (1.3)	21.5 (1.4)
	III	20	0.1	6	27.2 (3.1)	21.8 (2.7)	19.9 (2.4)	22.4 (2.0)	23.1 (2.2)	22.7 (2.2)	21.4 (2.4)	21.7 (2.5)	21.9 (2.9)	21.5 (3.2)	21.0 (3.3)	21.1 (3.0)	19.0 (2.5)	19.2 (2.3)	18.5 (2.5)	18.1 (2.4)	18.8 (2.5)
	IV	20	0.215	6	27.4 (2.8)	21.6 (1.6)	21.1 (2.0)	21.0 (2.2)	22.2 (2.0)	22.7 (2.1)	21.9 (2.1)	21.0 (2.1)	20.5 (2.5)	19.9 (3.0)	21.7 (3.6)	20.1 (3.3)	20.8 (3.3)	19.8 (3.2)	18.6 (3.1)	19.3 (2.8)	19.1 (2.8)

Table 6: HAEMODYNAMIC VARIABLES / HR and MABP x HR / Dog / LCX
Dogs were subjected to 6 h LCX thrombotic occlusion (group I), or thrombotic occlusion followed by infusions, starting at t = 1.5 h, of r-scu-PA (30 min duration, groups II, III and IV) together with taprostene (TAP) (2 h infusion) at low dose (group III), high dose (group IV) or taprostene vehicle (group II). HR = heart rate (beats/min). MABP x HR = heart rate pressure product (metres Hg/min) as an estimate of myocardial work and hence energy demand.
All values arithmetic mean ± standard error (x ± S.E.M.) No statistical difference was observed between groups: Student's t-test, 2-tail, unpaired data.

BASELINE CHARACTERISTICS AND INFARCT PARAMETERS / Dog Infarct LCX

Group	CK_{max} (Ul^{-1})	I/LV (%)	I/AaR (%)	Aar/LV (%)	Sex (% male)	Weight (kg)	Thrombolysis Time (min)
I	729 (6)	23.1	50.2	46.0	m	15.3	> 360
	978 (6)	30.1	68.1	44.2	m	14.0	> 360
	557 (6)	20.6	43.2	47.7	m	18.0	> 360
	799 (6)	9.9	31.5	31.4	f	16.0	> 360
		20.9±4.2	48.3±7.7	42.3±3.7	(75)	15.8±0.8	-
II	2934 (6)	20.6	43.2	47.7	m	16.7	27
	2903 (6)	3.4	8.4	40.5	f	16.7	20
	2945 (6)	12.1	32.7	37.0	f	19.8	22
	1425 (6)	6.5	17.5	37.1	m	15.5	14
	1294 (6)	14.5	35.9	40.4	m	17.5	27
	1862 (6)	5.8	18.0	32.2	m	17.6	25
	1483 (6)	10.6	32.0	33.1	m	17.0	20
	1766 (6)	7.1	17.3	40.9	f	16.0	31
	1380 (6)	8.2	22.6	36.3	m	17.2	12
		9.9±1.8	25.3±3.7 *	38.4±1.6	(67)	17.1±0.4	22.0±2.1
III	7152 (6)	20.8	44.6	46.7	f	17.7	25
	1641 (6)	8.9	21.8	41.0	f	19.8	17
	547 (6)	2.9	8.3	35.2	m	15.0	21
	337 (6)	4.2	12.2	34.1	m	18.3	14
	1485 (6)	16.5	35.7	46.1	m	18.2	17
	337 (6)	1.7	5.2	33.0	m	16.5	15
		9.2±3.2	21.3±6.5 *	39.4±2.5	(67)	17.6±0.7	18.2±1.7
IV	399 (6)	5.7	14.4	39.5	m	15.1	12
	673 (5)	5.1	19.2	26.3	m	18.0	27
	1809 (5)	8.5	25.2	33.4	m	16.0	20
	631 (6)	2.6	6.7	38.9	m	13.6	22
	631 (6)	3.8	9.0	42.2	m	13.8	22
	946 (6)	11.0	27.9	39.4	f	19.0	18
		6.2±1.3	17.1±3.5 **	36.6±2.4	(84)	15.9±0.9	20.2±2.0

table 7: **BASELINE CHARACTERISTICS AND INFARCT PARAMETERS / Dog Infarct LCX**
Showing individual variables (weight, sex) and infarct parameters (size relative to left ventricle (I/LV), to risk area (I/AaR), and proportion of LV "at risk" (AaR/LV) and maximum observed plasma creatine kinase activity (in brackets: time of observed maximum: hours after initial occlusion) as well as the thrombolysis time (= time from start of infusions to first LCX reflow).
Gp I: 6 h LCX thrombotic occlusion; r-scu-PA infused 90 min after LCX thrombosis in gps II, III and IV. Gps III and IV: low and high dose taprostene cotreatment respectively.
Mean of group values ± S.E.M.
* $p<0.05$, ** $p<0.01$ versus group I, Student's t-test, 2-tail, unpaired data

ARRHYTHMIA / Dog LCX Infarct

			TIME INTERVAL [h]						
gp	n	-	PIV	0 - 1	1 - 1.7	1.7 - 3	3 - 4	4 - 5	5 - 6
I	4	x	0	0.6	1.7	0.1	0.3	1.8	3.5
		S.E.M.	-	(0.4)	(1.3)	(0.1)	(0.1)	(0.8)	(1.4)
		range	0	0-5	0-20	0-1	0-2	0-8	0-17
		median	0	0	0	0	0	0	3
II	9	x	0	1.4	1.4	8.0	7.3	7.8	8.8
		S.E.M.	-	(0.4)	(0.3)	(1.1)	(1.0)	(1.1)	(1.2)
		range	0	0-6	0-8	0-20	0-20	0-20	0-20
		median	0	1	0	6	7	6	7
III	6	x	0	0.8	0.9	3.9	4.7	4.8	6.6
		S.E.M.	-	(0.3)	(0.3)	(1.2)	(1.2)	(1.3)	(1.3)
		range	0	0-7	0-5	0-18	0-18	0-18	0-18
		median	0	0	0	2	2	2	5
IV	6	x	0	0.5	0.5	6.3	3.4*	3.8*	4.5*
		S.E.M.	-	(0.2)	(0.2)	(1.5)	(1.0)	(0.8)	(1.1)
		range	0	0-2	0-4	0-20	0-16	0-16	0-20
		median	0	0	0	4	1	3	4

table 8: ARRHYTHMIA / Dog LCX Infarct
Mean (± S.E.M), median and range of frequency of ventricular ectopic beats (per 20 beats, recorded over 4 periods/interval/dog), in dogs undergoing LCX thrombotic occlusion at t = 0, followed by infusions starting at t = 1.5 h of vehicle (group I), r-scu-PA (groups II, III and IV) and taprostene (vehicle: gp II, low dose: gp III, high dose: gp IV; 2 h duration).
* = $p < 0.05$ vs gp II, 2-tailed t-test

PLASMA ASSAYS / Dog Infarct LCX

gp	[µg/kg/min] r-scu-PA	TAP	n	Time [h] PIV	0	1	2	2.5	3	4	5	6
AP												
I	0	0	4	100	99 (1)	98 (3)	95 (2)	100 (3)	98 (3)	98 (2)	105 (4)	105 (2)
II	20	0	9	100	97 (3)	102 (5)	95 (6)	93 (7)	83 (7)	87 (8)	82* (5)	82* (6)
III	20	0.1	6	100	98 (1)	97 (1)	95 (2)	84** (2)	78** (5)	88* (3)	81** (3)	82** (4)
IV	20	0.215	6	100	100 (3)	102 (2)	99 (4)	93 (3)	89 (5)	93 (4)	94 (6)	92** (3)
PLG												
I	0	0	4	100	99 (1)	95 (2)	97 (1)	97 (1)	97 (1)	98 (2)	100 (4)	98 (3)
II	20	0	9	100	98 (3)	105 (4)	97 (6)	92 (8)	86 (6)	97 (5)	86 (5)	82 (6)
III	20	0.1	6	100	100 (3)	101 (3)	99 (2)	94 (4)	86** (2)	91 (8)	84 (5)	78** (4)
IV	20	0.215	6	100	97 (3)	97 (3)	99 (6)	97 (6)	89 (7)	97 (2)	94 (3)	87* (3)
FIB												
I	0	0	4	120.8 (8.8)	120.0 (8.3)	110.8 (7.2)	110.3 (8.1)	124.3 (11.8)	115.3 (6.1)	117.3 (7.8)	114.3 (8.5)	116.8 (8.0)
II	20	0	9	113.9 (5.2)	112.6 (6.2)	112.6 (5.9)	109.9 (6.1)	100.9 (5.6)	98.0 (3.3)	100.8 (4.8)	102.2 (4.4)	107.0 (6.7)
III	20	0.1	6	165.7 (32.4)	159.0 (32.9)	161.8 (32.9)	162.8 (40.5)	154.5 (37.1)	144.7 (35.1)	139.8 (33.6)	150.2 (34.6)	153.7 (31.4)
IV	20	0.215	6	125.2 (11.5)	117.2 (9.9)	120.7 (9.7)	119.5 (11.8)	115.2 (10.5)	108.3 (11.6)	114.5 (9.3)	112.2 (8.8)	113.5 (10.1)
CK												
I	0	0	4	26 (5)	37 (4)	77 (4)	112 (15)	180 (22)	237 (9)	416 (55)	617 (24)	766 (87)
II	20	0	9	22 (4)	30 (5)	97 (16)	176 (39)	941 (362)	1055 (221)	1364 (259)	1571 (260)	1990 (242)
III	20	0.1	6	26 (5)	29 (6)	130 (59)	184 (58)	582 (192)	754 (258)	1436 (787)	1718 (981)	1917 (1073)
IV	20	0.215	6	19 (4)	28 (4)	59 (8)	145 (34)	306 (69)	455 (144)	565♦ (85)	771♦ (221)	750♦♦ (123)

table 9: PLASMA ASSAYS / Dog Infarct LCX
Dogs were subjected to 6 h stable LCX thrombosis (group I), or infusions, starting at t = 1.5 h after thrombosis, of r-scu-PA (30 min duration, groups II, III and IV) together with taprostene (TAP) (2 h infusion) low dose (group III), high dose (group IV) or taprostene vehicle (group II).
AP= α-2-antiplasmin activity; PLG= plasminogen activity (both as % of preischaemic value (PIV)), FIB= fibrinogen concentration (mg/100ml), CK= creatine kinase activity (U/l).
All values arithmetic mean ± standard error (x ± S.E.M.)
* = $p<0.05$, ** = $p<0.02$; Student's t-test, 2-tail, unpaired data, versus group I
♦ = $p<0.05$, ♦♦ = $p<0.02$; Student's t-test, 2-tail, unpaired data, versus group II

3.3 Rabbit carotid artery thrombosis experiments/Results

The experimental protocol is represented diagrammatically (diagram 13, "Methods" section)

3.3.1 Haemodynamic parameters/Rabbit

Mean arterial blood pressure (MABP)/Rabbit

The 4 group mean MABP values were similar prior to treatment (-20 and -10 min) with the test compounds. During this interval the group mean values ranged from 68.8 ± 4.8 mm Hg ($\bar{x}$ ± S.E.M.) (-10 min, randomized group later to receive taprostene) to 79.8 ± 1.3 mm Hg (-20 min, later to receive r-scu-PA). Before starting the infusions, the individual MABP values ranged from 54.3 to 82.6 mm Hg. At t = -10 min the infusions were started. In the 10 minutes thereafter minimal change in MABP was observed. The dose of taprostene (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$) had been chosen from preliminary experiments as the highest dose in a series of stepwise increments not exerting a significant hypotensive effect in pentobarbitone anaesthetized rabbits (n = 5). The stenosis was implanted into the carotid artery at t = 0 min, immediately prior to this no significant difference between the group mean MABP values ($\bar{x}$ ± S.E.M.) was recorded (range of group means ± S.E.M.: 69.9 ± 3.8 mm Hg in the taprostene treated group to 80.8 ± 2.6 mm Hg in the r-scu-PA treated group).

The presence of the unilateral stenosis had no major effect on the MABP in any of the groups, neither did the infusion of taprostene (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v. 100 min duration, starting 10 min before stenosis, group III, n = 5) or the combination of taprostene and

r-scu-PA infusions (group IV, n = 5). The marginally higher MABP values in the r-scu-PA group (group II) became significantly different to the r-scu-PA untreated group values ($p < 0.02$, t-test, 2-tail vs. group I) after completing the infusion. The mean MABP values in the r-scu-PA group were consistently higher for the duration of the experiment, including the pretreatment phase and is therefore not definitely attributable to an effect of this compound. In summary the mean arterial blood pressure was not affected by the following factors: carotid artery stenosis, taprostene infusion and taprostene and r-scu-PA combination treatment. r-scu-PA infusion may increase the blood pressure marginally in this model (table 10, diagram 34).

Carotid artery flow rate/Rabbit

The normal pretreatment carotid artery flow rates ($ml \cdot min^{-1} \cdot kg^{-1}$ bodyweight) showed moderate variation: the group means (± SEM) ranged from 7.5 ± 0.8 (group III) to 10.6 ± 1.2 $ml \cdot min^{-1} \cdot kg^{-1}$ (group IV) and the range of individual values was between 4.7 and 15.7 $ml \cdot min^{-1} \cdot kg^{-1}$ at this time point (-20 min). After 10 minutes equilibration the group flow rates showed little change and the infusions were started. Implantation of the stenosis (t = 0 min) was accompanied by a marked fall in the flow rates. Here the group mean values (± S.E.M) were 5.3 ± 0.7 $ml \cdot min^{-1} \cdot kg^{-1}$, 5.4 ± 0.8 $ml \cdot min^{-1} \cdot kg^{-1}$, 5.8 ± 0.9 $ml \cdot min^{-1} \cdot kg^{-1}$ and 7.2 ± 1.1 $ml \cdot min^{-1} \cdot kg^{-1}$ for the control-, taprostene-, r-scu-PA- and taprostene plus r-scu-PA-treated groups (I - IV) respectively (diagram 35).
This equated in individual animals to a 30 - 50% reduction compared with normal non-stenotic flow. In the control rabbits the mean flow rate decreased dramatically; 1 of the 6 animals occluded within 5 minutes, 4 within 10 minutes and 5 within 30 minutes. Spontaneous reflow and reocclusion occured in some of these animals but was relatively infrequent as occlusion tended to predominate (diagram 37). One animal showed subtotal (1.1 ml/min x kg) occlusion at 15 min followed however by a slow increase in flow rate and subsequent reduction.

The rabbits treated with r-scu-PA (21.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v. 40 min duration, starting 10 min prior to stenosis, group II, n = 5) showed a significantly higher stenotic flow rate compared with the

controls ($p < 0.05$, t-test, 2-tailed) at 10 and 15 min after stenosis and approximately 30 min after discontinuing the r-scu-PA infusion (t = 60 min) the mean flow rate started to decline and continued to do so over the remaining 2 hour period approaching the control value (diagram 35, table 10). During the period of r-scu-PA infusion, and afterwards, total and subtotal occlusion occured. Spontaneous restoration or increase in the stenotic flow rate was observed more frequently here than in other treatment groups.

In a third group, taprostene infusion (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v., 100 min duration, starting 10 min before stenosis, group III, n = 5) caused the stenotic flow rate to be maintained over the 3 hour experimental period at approximately 50 % that of the prestenotic rate after an initial lower fall. However, due to much interanimal variation this was not statistically greater than the mean flow rate in the vehicle treated group until 150 and 180 min after introduction of stenosis. Interestingly, the taprostene infusion had been stopped prior to this time.

In group IV, rabbits (n = 5) were treated with both r-scu-PA and taprostene as in groups II and III. In these animals the fall in flow rate following stenosis was much attenuated compared to the separate treatment groups and more so compared to the vehicle-treated controls. The non-stenotic flow rate was maintained at 60 - 80 % of the normal non-stenosic flow rate.

The measurement of instantaneous flow rate as done above was accompanied by major drawbacks. In preliminary experiments to establish this model a "saw toothed" flow pattern was seen occasionally in some animals. This unstable cyclical flow pattern limits the value of flow rate data at fixed time points. For this reason a means of measuring the total volume of blood flow over certain intervals was developed; by electronically converting the flow-meter potential difference into counting rate, and then using a count rate/flow rate calibration curve, it was possible to determine the mean flow rate (adjusted for animal body weight) during the specified intervals ($ml \cdot min^{-1} \cdot kg^{-1}$ over the specified interval = mean interval flow rate).

As only very mild fluctuations in flow were observed in all animals prior to stenosis implantation the interval mean flow rates were similar to the mean flow rates at fixed time points (-20 and -10 min) (compare diagrams 35 and 36). The baseline mean carotid flow rates (interval -20 to -10 min) were subject to variation between animals (range 4.9 to 13.2 $ml \cdot kg^{-1} \cdot min^{-1}$), but the group IV means were comparable with one another (diagram 36, table 11). For the fraction of the infusion periods prior to stenosis (-10 to 0 min) the group means were also comparable and virtually unchanged, implying that the carotid flow rates were unaffected by r-scu-PA and taprostene alone and together, in the absence of an arterial stenosis.

During the 15 minute period starting with the implantation of the stenosis, the interval mean flow rates were attenuated to different extents. Considering the group mean values, a ≈ 90 % fall in the mean interval flow rate was seen in the vehicle treated group (group I) after stenosis. The mean value for this group remained at this level until decreasing towards the end of the 3 hour stenotic period (diagram 36, table 11).

In the groups treated with either taprostene alone (group III) or r-scu-PA alone (group II) the mean interval flow rates did not fall to the same extent as in the vehicle treated group, either initially, or at any interval in the observation period. Stenosis caused an approximate 50 - 60 % attenuation of mean interval flow rates in both these groups during the first 15 min interval.

During the 30 min interval following cessation of the r-scu-PA infusion (30 - 60 min) the flow rates were significantly increased above the corresponding control group mean values. After cessation of the r-scu-PA infusion, however, the flow rates decreased consistently with time.

In the taprostene-alone treated group, the flow rose slightly after an initial post-stenotic flow (40 % of non stenotic) to above 60 % of basal. In this group the flow was maintained at this level for the duration of the observation period, that is 90 minutes after stopping the taprostene infusion.

Cyclical flow reduction (CFR)

Cyclical flow reductions are defined in the "Methods" section and recorded from the flow paper-trace. Prior to stenosis no cyclical flow reduction (CFR) was observed. The infusion of the test compounds, starting 10 minutes before stenosis did not cause flow variations (CFR).

In the vehicle treated group, stenosis was accompanied by occlusion in most cases (see "Carotid artery flow rate"/Results). Spontaneous increases in flow rate did occur but were infrequent (table 11 diagram 37). r-scu-PA infusion (group II) led to a marked increase in the number of CFR events occuring. These were relatively evenly distributed between the members of the group and over the experimental period of stenosis.

Infusion of taprostene together with r-scu-PA (group IV) totally abolished the CFR ($p \leq 0.05$, Chi squared test) seen with r-scu-PA alone except during the last 30 minute observation period (150 - 180 min) when a total of 3 CFR events were seen in 2 of the 5 animals in the group. Taprostene, on its own (group III), also abolished CFR ($p \leq 0.05$) compared to the r-scu-PA group except for during the initial 15 minute period after implantation of the stenosis (diagram 36, table 11).

3.3.2 Haemostatic parameters/Rabbit

Fibrinogen in plasma/Rabbit

Clottable fibrinogen was measured with an automated Clauss assay using human thrombin. The fibrinogen concentration in plasma immediately prior to infusion of the test compounds (-10 min) showed extreme consistency in groups I - III (range of group means ± SEM = 287 ± 15 (gp I) to 301 ± 11 mg/dl (gp III)), whereas in the combination treatment group (r-scu-PA + taprostene (gp IV)) a higher relative group mean fibrinogen level was observed (356 ± 29 mg/dl) prior to infusion. This was attributed to one rabbit in this group which had a consistently higher level of fibrinogen (462 mg/dl at -10 min and throughout the experiment) but the data was nevertheless included in the experimental results (diagram 38, table 12). After 60, 120 and 180 minutes, the plasma fibrinogen values remained virtually unchanged in all treatment groups when compared with the baseline values of the respective groups. Taprostene and r-scu-PA infusion, alone and together, therefore had no effect on clottable fibrinogen in plasma under these experimental conditions.

Platelet aggregation/Rabbit

As this assay was included in the experimental protocol after the first few experiments had been completed, platelet assays were not performed for all animals.

Immediately prior to starting the infusions (t = -10 min) of taprostene and/or r-scu-PA and/or vehicle the % platelet aggregation after collagen stimulation in vitro was similar in all animals. End concentrations of 5 µg/ml collagen caused ≈ 75 % aggregation (range 58 to 91 %; n = 17 animals). Collagen at 2 µg/ml caused ≈ 40 % aggregation (range 14 to 76%) at this time, and 1 µg/ml caused approximately 15 % aggregation (range 48 to 0%). Samples from 2 to 3 of the 4 to 5 animals per group failed to respond to 1 µg/ml collagen at t = -10 min as it was a subthreshold concentration.

After 40 min of taprostene infusion (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$, t = 30 min) mean platelet aggregation was attenuated by 28 % after 5 µg/ml collagen stimulation compared to the vehicle group (diagram 39, table 12).

At this time (t = 30 min) there was seen a relative mean attenuation of platelet aggregation of 50% at 2 µg/ml collagen compared to the vehicle group. The response to 1 µg/ml collagen was absent in all 4 taprostene treated animals but present in 2 of 4 vehicle group animals (62 and 17% aggregation respectively at the same time point (t = 30 min). These inhibitory effects of taprostene failed to achieve statistical significance due to the small numbers of animals tested but the distinct trends are considered relevant however. Ninety minutes after termination of the taprostene

infusion (t = 180 min) the mean % aggregation had risen again to that seen in the vehicle group (≈ 45%) after stimulation with 5 µg/ml collagen (range in taprostene group: 0 to 84%, in vehicle group: 42 to 50%). Thus during taprostene infusion the mean aggregatory response was reduced compared to vehicle infusion and the threshold concentration of collagen required to produce aggregation may have been elevated. The aggregatory response recovered within 90 min of terminating the taprostene infusion (t = 180 min) (diagram 39, table 12).

Infusion of r-scu-PA alone (21.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 40 min duration) had no effect at all on the mean % aggregation compared to vehicle treated controls, after stimulation with 5 and 2 µg/ml collagen but the threshold concentration of collagen required to cause aggregation may have been slightly raised by r-scu-PA. The mean % aggregation in vehicle and r-scu-PA treated animals was similar 150 min after termination of the r-scu-PA infusion (t = 180 min). Thus platelets from r-scu-PA treated animals behaved similarly to those from vehicle treated animals apart from a possible slight increase in the collagen threshold concentration (diagram 39, table 12).

Samples from 4 animals that received combined infusions of r-scu-PA and taprostene (composite of 2 infusion regimens) were available for the platelet assay. 40 minutes after starting both infusions together (t = 30 min) the effect of 5 µg/ml collagen on platelet aggregation was very similar to taprostene treatment alone, and the mean % attenuation compared to vehicle treatment is attributable therefore to taprostene alone.

In this combination r-scu-PA/taprostene group at t = 180 min the mean aggregation was 36 % lower than that seen in the taprostene

alone group. In both groups, samples from 1 of the 4 animals did not respond to 5 µg/ml collagen, whereas 2 µg/ml caused aggregation in 1 of 4 taprostene group samples but none of 4 r-scu-PA/taprostene group samples. The number of platelet rich plasma samples available for assay in this study was inadequate to provide refutable proof of a prolonged potentiating effect of r-scu-PA on taprostene inhibition of platelet aggregation, although there is limited evidence of this. Worthy of comment is the increase over time in the collagen concentration threshold causing aggregation and the decrease in maximum aggregation response in all groups (diagram 39).

r-scuPA may not have a potentiating effect on the inhibition by taprostene of in vivo platelet aggregation after collagen stimulation under these conditions. Additionally, there was no evidence of r-scu-PA-induced platelet hyperaggregability after 40 min of in vivo infusion and in vitro stimulation of platelet aggregation with collagen. In contrast, a slight elevation of the collagen threshold concentration required to produce platelet aggregation was observed, but this effect was common to the r-scuPA untreated groups as well.

Summary of results from rabbit carotid artery stenosis experiments

To summarize the rabbit carotid artery stenosis experiments, in vehicle treated animals implantation of the unilateral stenosis caused thrombotic occlusion in the majority of animals, with very little spontaneous reopening. Treatment with a r-scuPA infusion (21.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 40 min) temporarily increased the flow rate through the stenosis and delayed occlusion (diagram 35, 36, 37). Taprostene infusion at a non hypotensive dose (0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 100 min (diagram 34)) also increased the mean flow rate through the stenosis and delayed its occlusion. Combining infusions of taprostene and r-scu-PA caused a potentiated effect. Stenotic flow rate was maintained at a rate higher than in the separate treatment groups and no occlusions occurred over the observation period. Taprostene acted by attenuating the cyclical decreases in the carotid artery flow rate (diagram 37) reported to be caused by platelet aggregates, and in vitro collagen-induced platelet aggregation showed a possible inhibitory effect of taprostene at this dose level although the number of animals studied was small (diagram 39). Treatment with r-scuPA either alone or with taprostene did not exert an antithrombotic effect by degradation of circulating fibrinogen as in neither group was the concentration of fibrinogen (Clauss assay) changed after treatment (diagram 38). Cotreatment of taprostene with r-scuPA also completely abolished the cyclical flow variations seen in the r-scuPA alone group, an action attributed to inhibition of platelet aggregation by taprostene as seen again in the combined treatment group. Treatment with r-scuPA did not appear to either increase or decrease platelet

aggregability as measured in vitro although a high frequency of cyclical flow variations through the stenosis was recorded (diagram 37) possibly due to a continuous destabilization of the occlusive thrombus. There was a delayed trend to increased mean arterial pressure in this group (diagram 34).

Mean Arterial Blood Pressure (MABP)/Rabbit

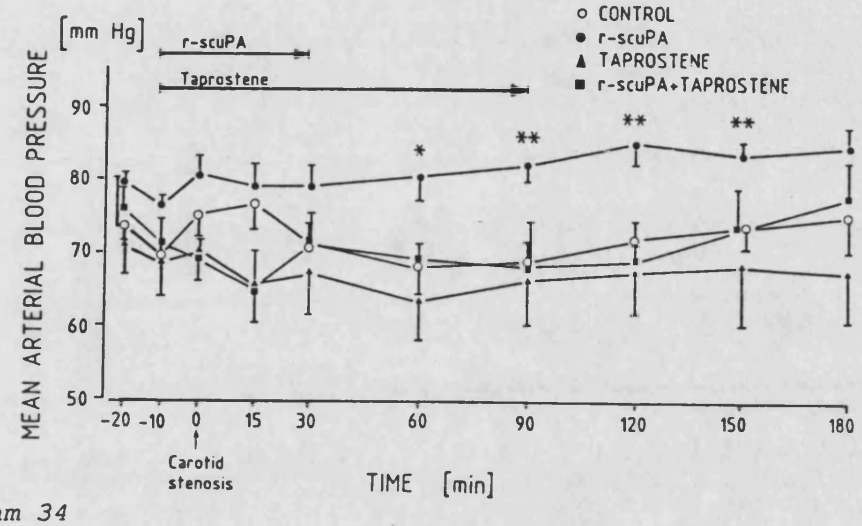


Diagram 34

Carotid Artery Stenosis Flow Rate/Rabbit

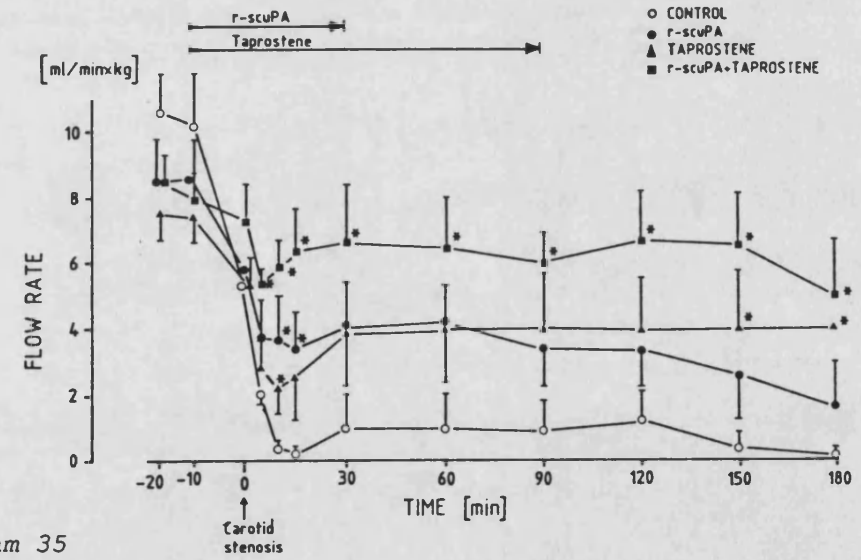


Diagram 35

Diagrams 34 and 35: Mean arterial blood pressure (MABP) [mm Hg] and mean carotid artery flow rate [ml.min^{-1}.kg^{-1}] at specified time points. Rabbits with carotid artery stenoses, (onset t = 0h) were treated, starting at t = -10min, with: group I - vehicle (n=6), II - scu-PA (21.5 µg kg^{-1} min^{-1}, 30 min duration, n=5), III: taprostene (0.215 µg kg^{-1} min^{-1}, 2h duration, n=5) or IV: combined infusions (taprostene and r-scu-PA each as above, n=5) ($\bar{x}$ ± S.E.M.).

** = p<0.05, ** = p<0.02 vs group I, t-test, 2-tailed, unpaired data.*

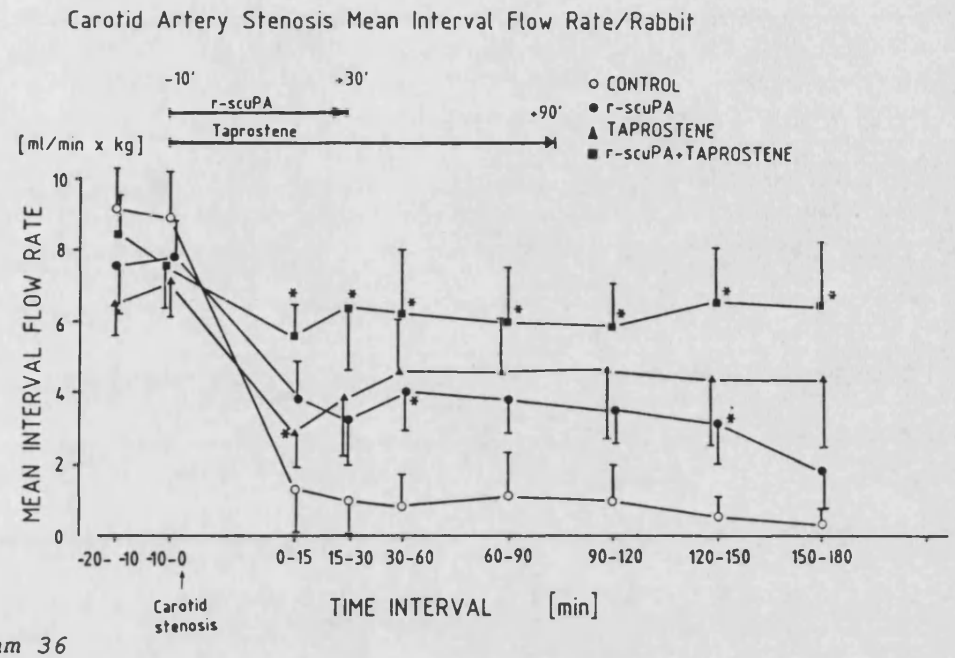


Diagram 36

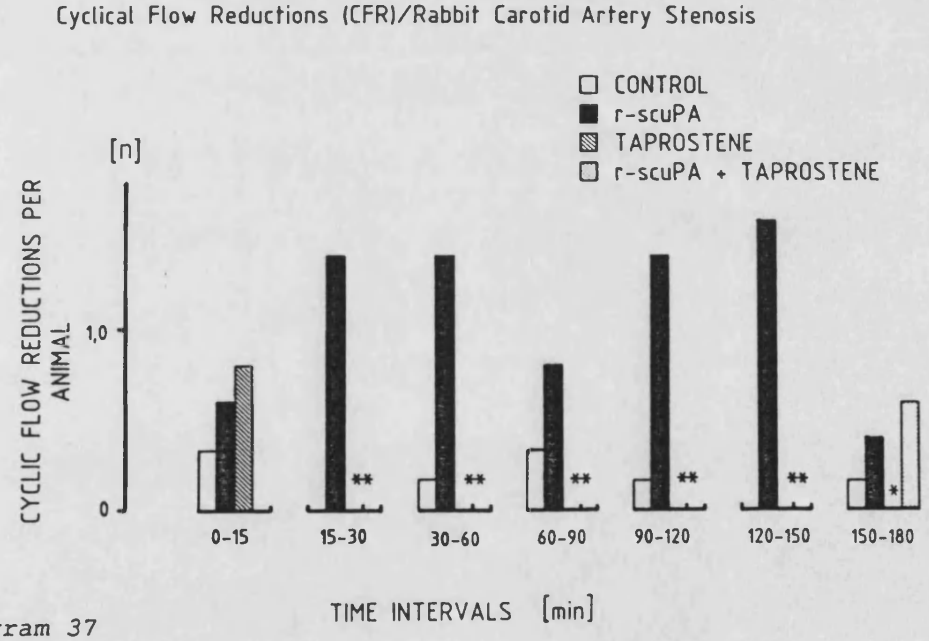


Diagram 37

Diagrams 36 and 37: Mean interval flow rate [ml . min^{-1} . kg^{-1}] ($\bar{x}$ ± SEM) and frequency of cyclical flow reduction (CFR) (see "methods") both over the time inverals specified. Rabbits were treated as outlined in legend to tables 34 and 35, and methods.
** = $p<0.05$, for flow rate (diagram 36): t-test, 2-tail, unpaired data versus group I (controls: hollow circles); for CFR (diagram 37): * = $p<0.05$, Chi squared test, versus group II (r-scu-PA alone: blacked out columns).*

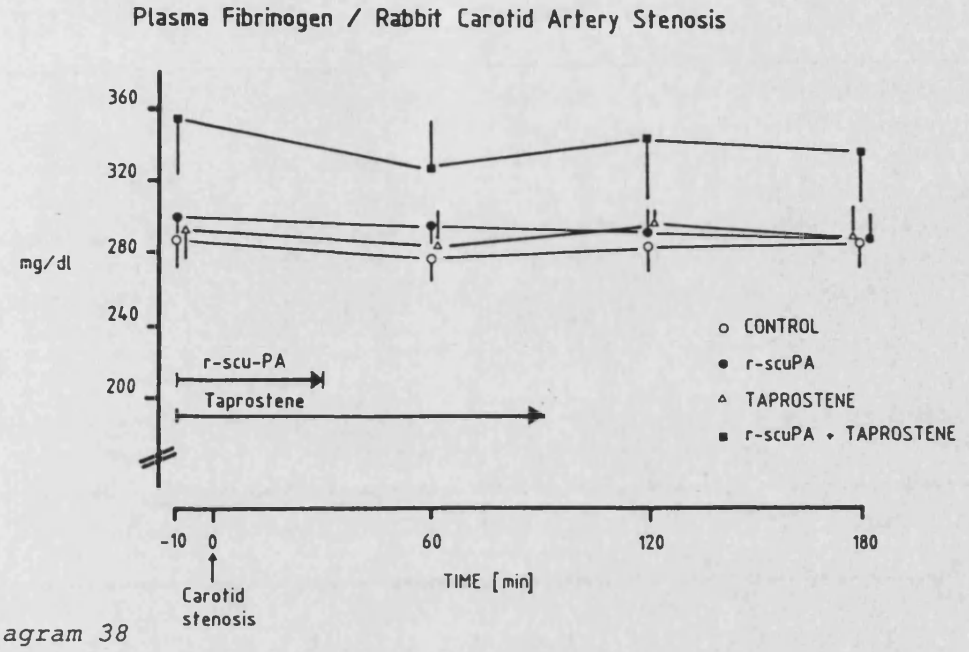


Diagram 38

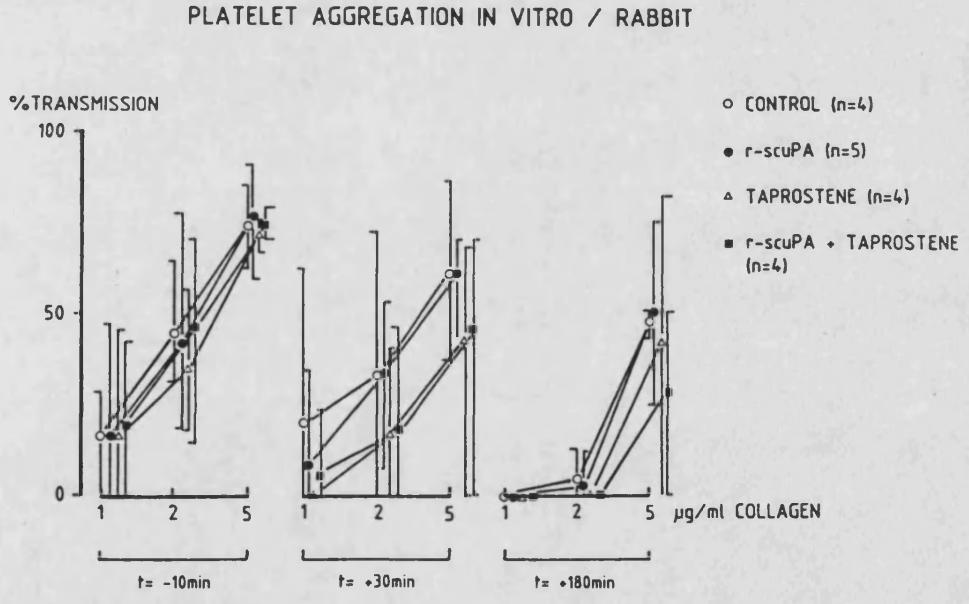


Diagram 39

<u>Diagrams 38 and 39:</u> Plasma fibrinogen concentrations [mg/dl] and in vitro collagen-induced aggregation in platelet-rich plasma [% of light transmission compared to platelet poor plasma, after collagen stimulation, Born aggregometer]. For diagram 38: $\bar{x} \pm$ S.E.M.; diagram 39: $\bar{x} \pm$ absolute range of values. Treatments are described in "methods". Fibrinogen levels were not altered by drug treatments.

HAEMODYNAMIC VARIABLES / Rabbit Carotid Artery Stenosis

gp	[µg/kg/min] r-scu-PA	[µg/kg/min] taprostene	n	Time [min] - 20	- 10	0	5	10	15	30	60	90	120	150	180
				MEAN ARTERIAL BLOOD PRESSURE (MABP) [mm Hg]											
I	0	0	6	73.5 (6.5)	69.2 (5.3)	74.9 (5.5)	74.8 (5.3)	76.5 (3.1)	76.8 (3.4)	71.4 (3.3)	68.2 (3.8)	68.9 (2.7)	72.3 (2.6)	73.6 (2.7)	75.2 (4.7)
II	21.5	0	5	79.8 (1.3)	76.8 (1.1)	80.8 (2.6)	75.8 (2.1)	77.4 (2.7)	79.0 (3.0)	79.0 (3.0)	80.5 * (3.1)	82.2** (2.2)	85.4** (2.6)	84.2** (2.2)	85.1 (2.4)
III	0	0.215	5	70.6 (5.3)	68.8 (4.8)	69.9 (3.8)	65.9 (5.1)	65.0 (5.9)	65.7 (5.4)	67.7 (5.7)	63.8 (5.2)	66.8 (5.8)	67.4 (5.5)	68.6 (8.0)	67.6 (6.2)
IV	21.5	0.215	5	76.1 (3.0)	71.3 (3.7)	69.5 (2.2)	69.3 (3.6)	68.7 (3.8)	67.3 (4.5)	71.3 (4.0)	69.0 (2.2)	68.5 (5.9)	69.1 (2.2)	74.1 (4.8)	78.0 (4.7)
				MEAN INSTANTANEOUS FLOW RATE [ml/min x kg]											
I	0	0	6	10.6 (1.2)	10.22 (1.6)	5.3 (0.7)	2.0 (1.0)	0.3 (0.2)	0.2 (0.1)	1.0 (1.0)	1.0 (1.0)	0.9 (0.9)	1.2 (0.9)	0.4 (0.4)	0.2 (0.2)
II	21.5	0	5	8.4 (1.3)	8.5 (1.2)	5.8 (0.9)	3.7 (1.1)	3.7 * (1.4)	3.4 * (1.2)	4.1 (1.4)	4.2 (1.1)	3.3 (1.1)	3.4 (1.1)	2.6 (1.3)	1.7 (1.3)
III	0	0.215	5	7.5 (0.8)	7.4 (0.7)	5.4 (0.8)	2.8 (0.9)	2.1 * (0.7)	2.5 (1.3)	3.9 (1.6)	4.0 (1.6)	4.0 (1.7)	3.9 (1.6)	4.0 * (1.7)	4.0 * (1.7)
IV	21.5	0.215	5	8.4 (0.8)	7.8 (1.0)	7.2 (1.2)	5.5 * (0.5)	5.8 * (0.8)	6.4 * (1.3)	6.5 * (1.8)	6.3 * (1.6)	5.9 * (0.9)	6.7 * (1.6)	6.5 * (1.6)	5.1 * (1.7)

Table 10: HAEMODYNAMIC VARIABLES / Rabbit Carotid Artery Stenosis
Mean arterial blood pressure (MABP) [mm Hg] and mean instantaneous carotid artery flow rate [ml/min x kg body weight]. r-scu-PA was infused (gps II and IV) from -10 min to +30 min; taprostene was infused (gps III and IV) from -10 min to + 90 min. The carotid artery was stenosed from t = 0 to t = 180 min.
* = $p<0.05$, ** = $p<0.02$ versus group I, Student's t-test, 2-tail, unpaired data.

HAEMODYNAMIC VARIABLES / Rabbit Carotid Artery Stenosis

gp	[µg/kg/min] r-scu-PA	[µg/kg/min] taprostene	n	Time [min] after start of stenosis: -20 - -10	-10 - 0	0 - 15	15 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180
				INTERVAL MEAN FLOW RATE [ml/min x kg]								
I	0	0	6	9.2 (1.1)	8.9 (1.3)	1.3 (1.2)	1.0 (1.0)	0.8 (0.8)	1.2 (1.2)	1.0 (1.0)	0.6 (0.6)	0.4 (0.4)
II	21.5	0	5	7.5 (1.4)	7.8 (1.0)	3.8 (1.1)	3.2 (1.2)	4.0* (1.1)	3.8 (1.6)	3.5 (0.9)	3.1* (1.1)	1.8 (1.0)
III	0	0.215	5	6.5 (0.8)	7.1 (0.9)	2.9* (0.9)	3.8 (1.6)	4.6 (2.0)	4.5 (1.5)	4.6 (1.9)	4.4 (1.9)	4.4 (1.9)
IV	21.5	0.215	5	8.4 (1.1)	7.4 (1.3)	5.5* (0.9)	6.3* (1.7)	6.2* (1.8)	6.0* (1.5)	5.8* (1.2)	6.5* (1.6)	6.4* (1.8)
				CYCLICAL FLOW REDUCTIONS (CFR) [total no. events/interval]								
I	0	0	6	0	0	2	0	1	2	1	0	1
II	21.5	0	5	0	0	3	7	7	4	7	8	2
III	0	0.215	5	0	0	4	0 ♯	0 ♯	0 ♯	0 ♯	0 ♯	0 ♯
IV	21.5	0.215	5	0	0	0	0 ♯	0 ♯	0 ♯	0 ♯	0 ♯	3

Table 11: HAEMODYNAMIC VARIABLES / Rabbit Carotid Artery Stenosis
Mean interval flow rate [ml/min x kg body weight] over the intervals specified above and cyclical flow reductions (CFR) [total no. events/interval]. The stenosis was implanted at t = 0 for the duration of the experiment.
r-scu-PA was infused (gps II and IV) from -10 min to +30 min; taprostene was infused (gps III and IV) from -10 min to + 90 min.
♯ = $p<0.05$ versus group II, Chi squared test
* = $p < 0.05$ vs. group I, 2-tailed t-test unpaired data

HAEMOSTATIC VARIABLES / Rabbit Carotid Artery Stenosis

gp	infusion ($\mu g \cdot kg^{-1} \cdot min^{-1}$)			FIBRINOGEN [mg/dl plasma]				
	r-scu-PA	TAP		n	-10 min	60 min	120 min	180 min
I	0	0		6	287 (15)	277 (13)	284 (14)	287 (14)
II	21.5	0		6	301 (11)	295 (10)	292 (13)	288 (14)
III	0	0.215		6	293 (16)	285 (18)	296 (7)	290 (16)
IV	21.5	0.215		6	357 (29)	328 (27)	343 (34)	337 (27)
				PLATELET AGGREGATION [%]				
	r-scu-PA	TAP	COLL µg/ml	n	- 10 min		+ 30 min	+ 180 min
I	0	0	1	4	6.8 (6.8)		19.8 (14.7)	0
			2		44.1 (7.3)		33.4 (17.2)	4.4 (3.0)
			5		73.5 (5.0)		60.5 (11.4)	47.0 (1.6)
II	21.5	0	1	5	16.1 (10.1)		6.9 (6.9)	0
			2		42.2 (10.7)		34.1 (9.7)	2.5 (2.5)
			5		75.8 (6.0)		59.5 (4.5.)	49.5 (12.2)
III	0	0.215	1	4	16.5 (10.9)		0	0
			2		34.6 (8.4)		16.7 (10.1)	1.4 (1.4)
			5		71.3 (1.8)		43.4 (15.7)	42.2 (17.5)
IV	21.5	0.215	1	4	19.8 (11.5)		6.0 (6.0)	0
			2		45.6 (12.0)		18.1 (11.2)	0
			5		73.9 (2.0)		46.1 (16.0)	27.2 (10.2)

Table 12: HAEMOSTATIC VARIABLES / Rabbit Carotid Artery Stenosis
Mean plasma fibrinogen concentration [mg/100 ml] and collagen-induced platelet aggregation in vitro [% change in light transmission) of platelet-rich plasma after addition of collagen (COLL) (see Methods)]. r-scu-PA and taprostene were infused together (gp IV), separateley (gps II and III) or substituted with saline infusions (gp I). The carotid artery stenosis was implanted at t = 0, for the remaining duration of the experiment. No statistical difference between groups were observed.

3.4 The effects of taprostene infusion on selected haemostatic parameters in healthy male volunteers/Results

In this blinded, 3-way crossover, placebo-controlled study the following haemostatic parameters were measured:
α_2-antiplasmin (AP), fibrinogen, fast acting plasminogen activator inhibitor (PAI-I), platelet factor 4 (PF4) and the euglobulin clot lysis time (ECLT). The methods are described in 2.2.4. The individual results were:

Antiplasmin/healthy volunteers

Prior to infusion of both doses of taprostene (80 and 400 µg i.v. over 2 hours) and of placebo, the photometrically determined plasma activities of AP (see 2.2.4.2) were very similar in all subjects (range 86 to 109.6 % of control plasma pool) prior to treatment the group mean antiplasmin activity was placebo: 94.6 ± 2.1 %; low dose taprostene: 95.1 ± 3.0 %; higher dose taprostene: 98.2 ± 3.3 % (mean percent activity relative to control plasma pool ± S.E.M.)
Plasma AP-activites remained unchanged in both taprostene-treated groups relative to the placebo group, in which a slight increase (≈ 7 %) was observed at the end the 25 hour observation period. Taprostene infusion therefore, under these conditions, had no effect on the endogenous inhibition of plasmin in plasma. (diagram 40, table 13).

Platelet Factor 4 (PF4)/healthy volunteers and plasminogen activator inhibitor 1 assay (PAI)

The purpose of the PF4 assay was to detect plasma samples in which ex vivo platelet activation had occurred. This is necessary as platelet alpha granules also contain high concentrations of PAI-1, hence activation in vitro would lead to spurious results. PF4 antigen level was measured (ELISA) in the same platelet free CTAD plasma supernatant used for the PAI-I activity assay.
Despite careful blood sample handling, 6 of the 180 plasma samples were rejected because of elevated PF4 levels (> 50 IU/ml taken as arbitrary cut-off point, the 6 values were all > 125 U/ml). All 6 rejected samples were found, after breaking the blinding code, to have originated in the placebo group. These samples were assayed for PAI-1 and distinctly elevated activities were recorded.
These 6 PAI-1 values were not included in the analysis. PF4 antigen levels tended to be higher before midday regardless of treatment and the lowest values in all groups were between 16.00 and 20.00 hours. This suggests a slight diurnal variation. The PF4 data were not further analyzed.

Fibrinogen/healthy volunteers

Baseline plasma fibrinogen concentrations ranged in total from 158 to 242 mg/100 ml. The treatment group means at this time were very similar (placebo group: 197 ± 12 mg/100 ml, low dose taprostene: 195 ± 10 mg/100 ml, higher dose taprostene: 203 ± 9 mg/100 ml ($\bar{x}$ ± S.E.M.)). The plasma fibrinogen levels remained very constant over the 25 h period in all 3 groups. Taprostene infusion for 2 h at these 2 dose levels therefore had no effect on this parameter (diagram 41, table 13).

ECLT/healthy volunteers

The rate of autolysis of thrombin-clotted plasma euglobulin fractions is the basis of a routine laboratory test (ECLT) employed here to quantify the relative fibrinolytic activity in vivo (see 2.2.4.5). The mean fibrinolytic rate (reciprocal of the lysis times: min^{-1}) was very similar in the 3 treatment groups at 8.00 a.m., prior to starting the infusions (group mean values S.E.M.: $2.8 \times 10^{-3} \pm 7.6\ 10^{-4}\ min^{-1}$; $2.4 \times 10^{-3} \pm 8.1 \times 10^{-4}\ min^{-1}$ and $3.3 \times 10^{-3} \pm 1.0 \times 10^{-4}\ min^{-1}$ for placebo, 80 µg/2 h and 400 µg/2 h groups respectively. Plasma from placebo treated volunteers exhibited a definite diurnal variation in the fibrinolytic activity with a nadir at or prior to 8 am and a maximum rate 2.5 times greater at 8 pm (diagram 43 table 13). The lower dose taprostene treatment group showed an earlier increase in fibrinolytic rate than the placebo group over the infusion period and lysis rates may have been marginally increased after infusion of taprostene in this group. The lysis rates remained slightly elevated over the placebo values for the remaining period. A more marked effect on the clot lysis rates was seen after high dose taprostene infusion, here the maximum mean rate was recorded at 10.30 am, 30 min after completing the infusion and therefore occurring much earlier than in the other 2 treatment groups (at 20 h). At this time point (t = 2.5 h) the lytic rate was increased by 167 % relative to the pretreatment value. This compared with corresponding increase of 122 % and 49 % in the low dose taprostene and placebo grops respectively. The clot lysis rates in the high dose taprostene group decreased slightly after the maximum at t = 2.5 h, converging slowly over an 8 hour period with the lysis rates in the low dose and placebo groups.

None of these effects reached statistical significance, but a distinct dose-related trend of increased clot lysis rates is implicated after taprostene infusion.

Summary of results from human study

To summarize the results from the taprostene infusion study in volunteers, neither of the infusion doses tested (80 µg/2h, 400 µg/2h) affected the plasma levels of α_2-antiplasmin or fibrinogen either during or up to 23 hours after completing the 2 hour infusions (diagrams 40 and 41). It may be possible that the infusions of taprostene decreased the activity of PAI-I in plasma during the infusion period but due to moderate intergroup variation in the pretreatment values this effect cannot be confirmed by the present experiments (diagram 42). The endogenous fibrinolytic rate as estimated by the euglobulin clot lysis assay (ECLT) demonstrated a distinct increase in the thrombolytic capacity of plasma during and up to 1 hour after the high dose taprostene infusion (diagram 43). The study design with small numbers did not have sufficient power to enable statistical significance of this effect to be attained however.

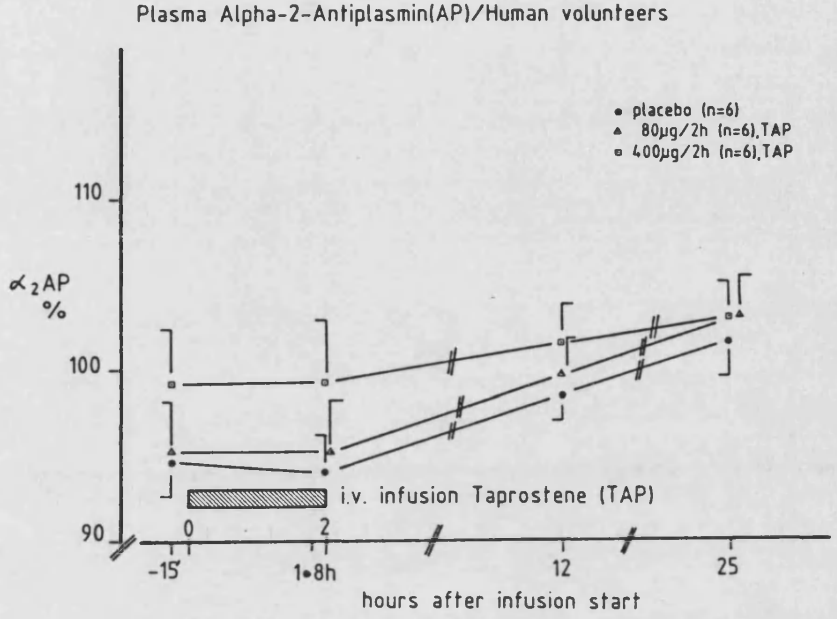


Diagram 40

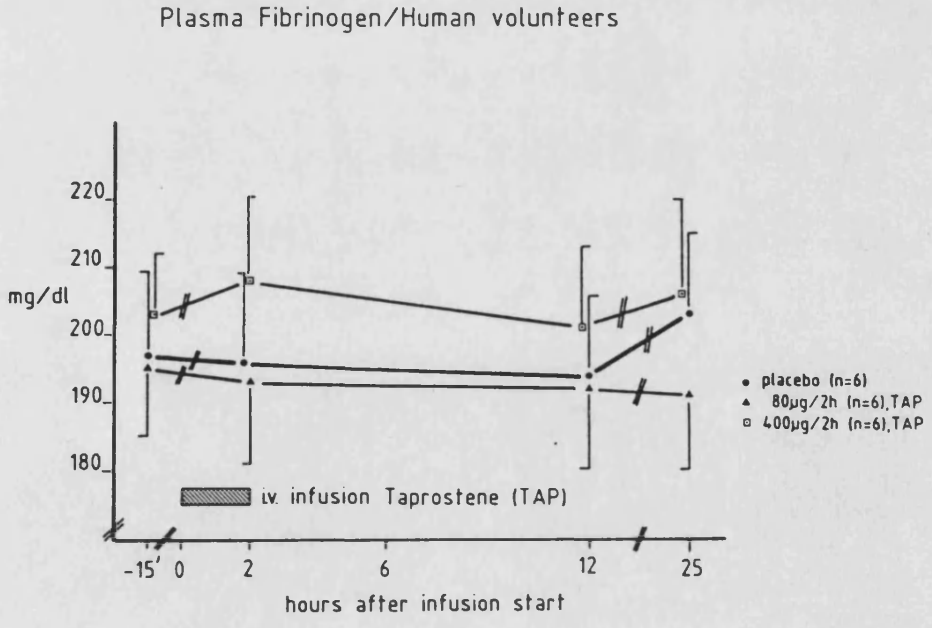


Diagram 41

Diagrams 40 and 41: Plasma α_2 antiplasmin activity (AP) [% of plasma pool] and fibrinogen concentration [mg/dl] ($\bar{x} \pm$ S.E.M.). Healthy male colunteers received infusions of placebo, or taprostene (80 µg/2h and 400 µg/2h) in a blind cross-over fashion. No significant difference was observed between any treatment at the same time points. O h corresponds to 8 am.

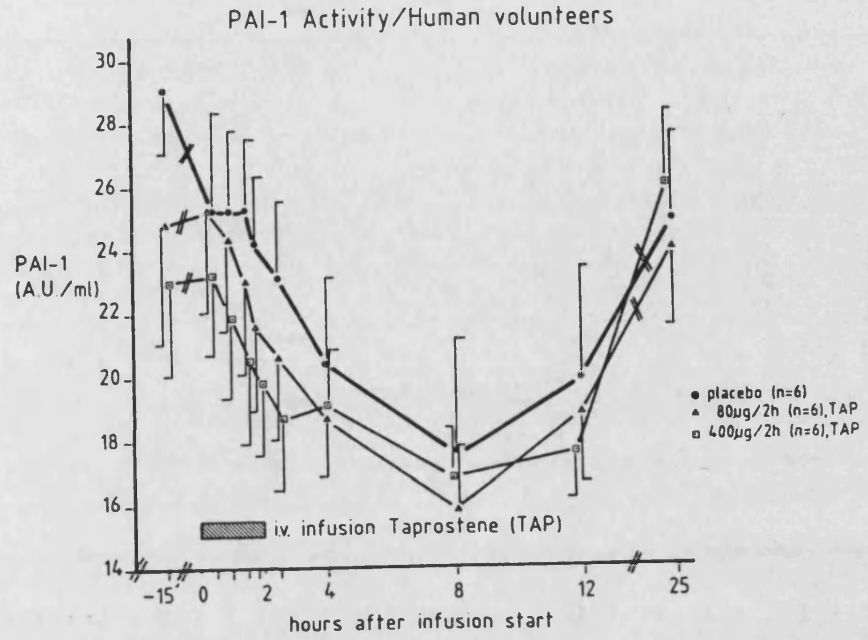


Diagram 42

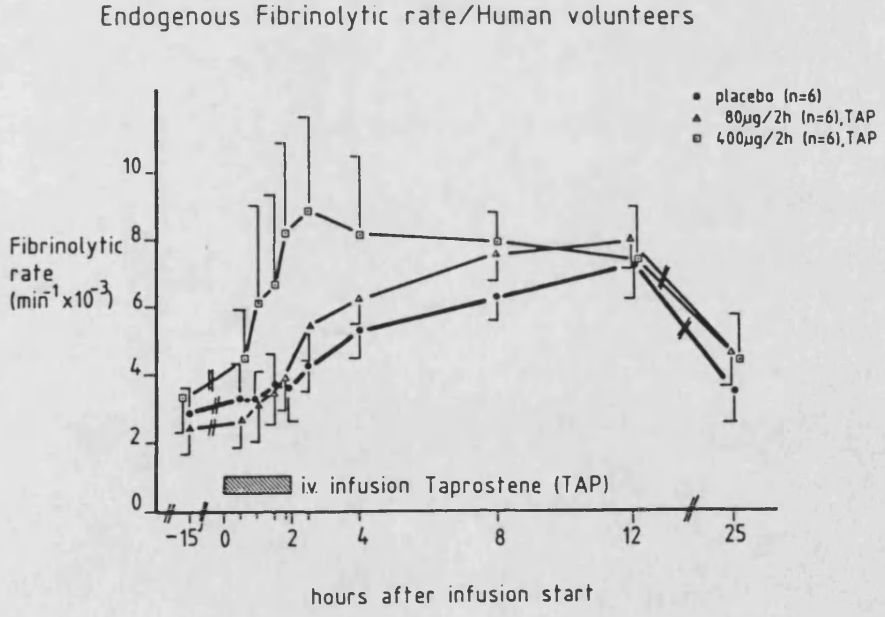


Diagram 43

<u>Diagrams 42 and 43:</u> Plasma activity of plasminogen activator-inhibitor-1 (PAI-1) [arbitrary units of tPA inhibition at 25°C (A.U.) per ml plasma] and endogenous fibrinolytic rate [reciprocal of euglobulin clot lysis times in min]. Healthy male volunteers received i.v. infusions of placebo, or taprostene (80 µg/2h) and 400 µg/2h) in a blind cross-over fashion ($\bar{x} \pm$ SEM) Time 0 = 8:00 am.

PLASMA ASSAYS / Healthy Male Volunteers

TAP i.v. infusion (µg/2h)	TIME [h] (pre) -0.25	0.5	1.0	1.5	1.8	2.5	4.0	8.0	12.0	25.0
	α_2-ANTIPLASMIN (%)									
placebo	94.6 (2.1)	-	-	-	94.0 (2.2)	-	-	-	98.5 (1.5)	101.7 (2.0)
80	95.1 (3.0)	-	-	-	95.2 (3.0)	-	-	-	99.7 (1.9)	103.5 (2.4)
400	98.2 (3.3)	-	-	-	98.3 (3.7)	-	-	-	102.6 (2.3)	103.0 (2.1)
	FIBRINOGEN (mg/100 ml)									
placebo	197 (12)	-	-	-	196 (13)	-	-	-	194 (12)	203 (12)
80	195 (10)	-	-	-	193 (12)	-	-	-	192 (12)	191 (11)
400	203 (9)	-	-	-	208 (12)	-	-	-	202 (12)	206 (14)
	PAI-1 (A.U./ml)									
placebo	28.9 A (2.0)	25.2 (3.1)	25.0 B (2.0)	27.3 B (2.7)	24.2 (2.1)	23.1 (2.4)	20.4 (2.7)	17.6 (3.4)	19.9 (3.4)	24.9 B (2.7)
80	26.2 (3.4)	25.5 (3.2)	24.3 (2.9)	23.0 (3.0)	21.6 (2.7)	20.6 (2.6)	18.7 (1.8)	15.8 (1.9)	18.8 (2.2)	24.0 (2.5)
400	23.6 (3.0)	23.2 (2.6)	21.9 (2.6)	20.5 (2.7)	19.8 (2.3)	18.7 (2.3)	19.1 (1.7)	16.8 (1.5)	17.6 (1.5)	26.0 (2.3)
	EUGLOBULIN CLOT LYSIS RATE (/min x 10^3)									
placebo	2.8 (0.8)	3.3 (1.0)	3.2 (0.8)	3.5 (1.0)	3.4 (0.9)	4.2 (0.7)	5.3 (0.8)	6.3 (0.6)	7.2 (1.0)	3.4 (1.0)
80	2.4 (0.8)	2.6 (0.8)	3.1 (1.1)	3.5 (1.0)	3.9 (1.0)	5.4 (1.0)	6.2 (0.7)	7.5 (0.8)	7.9 (0.8)	4.6 (1.0)
400	3.3 (1.0)	4.4 (1.5)	6.1 (3.0)	6.6 (2.7)	8.1 (2.7)	8.8 (2.8)	8.1 (2.3)	7.9 (0.8)	7.3 (1.6)	4.4 (1.4)

Table 13: **PLASMA ASSAYS / Healthy Male Volunteers**
Taprostene (2 dose levels and placebo) was infused intravenously (t = 0 to t = 2 h) in 6 healthy male volunteers using a blinded, randomised, cross-over protocol. α_2-antiplasmin activity = % of control plasma pool; fibrinogen: coagulation time assay; PAI-1 (plasminogen activator inhibitor-1): arbitrary units of tPA inhibition, PAI-1 samples with PF4 > 50 I.U./ml were rejected (A: n=1, B: n=2 rejected due to ex vivo platelet activation); Euglobulin clot lysis rate = reciprocal of euglobulin clot lysis time (min). All values x ± S.E.M.. No statistically significant difference between treatment groups; $p<0.05$, Student's t-test, 2-tail, paired data

4.0 Discussion

4.1 Dog infarct models

4.1.1 The model - a critique

In the initial series of experiments the LAD was chosen as the site of experimental thrombosis, but for the main series of infarction/ thrombolysis experiments the LCX artery was used. The reasons for the change over were numerous:

1) Anatomy: The high density of small bifurcations from the LAD, but not from the LCX, greatly hindered preparation and positioning of the flow probe.

2) Variability: The diameter of the LAD was smaller and more variable between animals than was the LCX. The size of the the LAD perfused bed (%Aar/LV) in the dog was found here to be smaller than that of the LCX (LAD: 15.3 ± 1.4% (range 7.9% to 23.8%; n=10) versus LCX: 38.8 ± 1.1% (range 26.3 % to 47.7%; n=25)), in agreement with Becker et al (1983) (LAD: 22.3 ± 1.5% versus LCX: 29.3 ± 2.0%, $p<0.01$). Gumm et al (Gumm, Cooper, Thompson et al. 1988) showed that proximal occlusion of the LAD and LCX resulted in %Aar/LV values of 43 ± 5 and 36 ± 2% respectively, whereas distal occlusion gave values of 13 ± 2% and 17 ± 2%. Thus, frequent proximal branching of the LAD imparts a different "arterial tree" pattern compared with the LCX, which causes greater variability in the former. Reimer, Ideker and Jennings (1981), by comparing different occlusion sites on the 2 arteries, had previously shown this difference.

Becker et al. (1983) concluded, after comparing risk areas (AaR/LV) of similar size, that LAD perfused beds were more severely ischaemic than LCX beds due to a lesser collateral supply, and hence, that canine LCX models would provide a more sensitive test of a drug's effect on infarction. This is especially important in the LCX experiments where there were 2 variables, treatment (taprostene or vehicle) and the duration of ischaemia. The duration of ischaemia was determined by the time taken to recanalize the vessel. In the LAD model more severe ischaemia would cause a more rapid and extensive evolution of necrosis. Therefore for a given variation in the duration of early ischaemia a greater variability in the relative infarct size of an LAD bed would result compared to an occluded LCX artery bed.

This possible effect was not confirmed retrospectively however in the LAD and LCX experiments. Comparing the relative infarct size after 2 hours ischaemia (group c (LAD) versus group II (LCX)), and after 6 hours ischaemia (groups a + b (LAD) versus group I (LCX)) showed that: (a) after 6 hours of ischaemia very similar proportions (≈ 50 %) of both anatomic risk areas were infarcted and, (b) after approximately 2 hours of ischaemia before thrombolysis the LAD infarcts were less extensive than the LCX infarcts (diagrams 22 and 28, table 7). This would imply that the severity of regional ischaemia is greater after LCX occlusion. The size of the occluded bed is however an important factor as the specific collateral flow rate (ml/min/g tissue) is inversely proportional to the size of the anatomic risk area (AaR) (Miura and Downey 1988; Gumm et al 1988).

3) Another reason for the change from LAD to LCX artery was the practicality /validity: A high proportion of LAD-occluded dogs dropped out of the analysis (29%) due to ventricular fibrillation occurring either during ischaemia or reperfusion. In an attempt to avoid this in a subset of LAD experiments lignocaine was given prior to ischaemia and during the acute recanalization phase. Despite successful attenuation of ventricular fibrillation (in all 4 dogs), this was later abandoned because lignocaine therapy has been shown to reduce various indices of infarction in canine models (Boudoulas, Karayannacos, Lewis et al. 1978; Nasser, Walls, Edwards et al. 1980) by 2 possible mechanisms, both common to PGI_2 - to stabilize myocardial membranes (Lesnefsky, van Benthuysen, McMurty et al. 1989) and to decrease neutrophil free-radical production (Peck, Johnston, Horwitz 1985). Thus any effect of taprostene here may have been concealed with concurrent lignocaine therapy. Only 1 of 30 dogs undergoing LCX occlusion (of which 21 underwent reperfusion) fibrillated.

A canine model of coronary thrombosis and thrombolysis was chosen because apart from primate models it seems to be the most comparable with man. Dog clots show the same order of sensitivity to lysis by scuPA in vitro as human clots do (Gurewich et al. 1984), and the pharmacokinetic profile of scuPA in vivo is similar ($t_{1/2}\alpha$ 6-9 min in both species (Van de Werf, Jang and Collen 1987; Van de Werf, Vanhaecke, De Geest et al 1986a)).

The canine haemostatic/fibrinolytic system is well characterized and the effect of various thrombolytic agents on coronary thrombi produced by differing methods has also been studied.

Taprostene shows a similar cardiovascular and antiplatelet profile in man and the dog ("Investigators' brochure" on taprostene supplied by Grünenthal GmbH). Although the general potency of taprostene may vary slightly between the 2 species, it is more important that the relationship antiplatelet/hypotensive potency does not vary greatly between the 2 species as the species difference can be compensated for by changing the infusion rate. It is probable that the difference between the dose of taprostene causing noticable antiplatelet action and that causing hypotension is narrower in anaesthetized models compared to conscious models and clinical situations as reflex compensation of hypotension is limited by anaesthesia.

Anaesthesia itself may have affected the infarct sizes. A single bolus of thiopental has been demonstrated to increase infarct size in a conscious canine model (Jugdutt, Rogers, Hutchins et al 1986). Deleterious effects of pentobarbitone on infarct size after 90 minutes canine coronary artery ligation and reperfusion had been described previously and were attributed predominantly to chronotropy (Mergner, Gilman, Patch et al 1985). In all experiments carried out in this present study the dose of anaesthetics used were relatively constant and would not have caused the marked differences in infarct sizes seen between certain treatment groups.

There are two further aspects of the model employed here that deserve critical consideration, the possible inter-animal variation in coronary collateral supply to the hypoperfused myocardium during artery occlusion and the staining technique employed to delineate

the relative infarct size and anatomic risk area. The magnitude of residual collateral flow to the myocardium after occlusion of the normally perfusing coronary artery is notoriously variable between animal species and even within a given species, including the dog or in man. In an unconscious canine LCX model of infarction the variation in the anatomic risk area was shown to account for 77% of the variation in infarct size relative to the left ventricular myocardium (I/AaR). After normalizing the infarct size to the area at risk, variation in collateral flow was found to account for 53% of the variability of the infarct size (Reimer, Jennings, Cobbs et al. 1985).

The determination of the collateral flow rate during occlusion in the present experiments by using radiolabelled microspheres was not possible due to a laboratory restriction of isotope use. As the only available countermeasures, ECG changes and visual-cyanosis of the myocardium on occlusion were used as criteria here to avoid inclusion of any animal with a well developed collateral system. Furthermore the animals were randomized to treatment, which was blinded until all data was available. The animals for this study were relatively inbred and homogeneous with respect to age and body weight in contrast to most published studies where it is evident that there is a high degree of heterogeneity. Therefore it is highly unlikely that differences in myocardial collateral flow in the different animals could have biased the infarct size results in the present experiments.

Considering the staining techniques employed to delineate the infarct size and area at risk, the methods employed have been shown to generate artefacts under certain conditions. After 90 minutes of

ischaemia followed by reperfusion the TTC dye used to stain viable tissue becomes reliably discriminative (Schaper 1984), however with no reperfusion non staining of microscopically-confirmed necrotic tissue only becomes reliable after 6 hours of occlusion, possibly because the limiting factor involved in the staining reaction, NAD cofactor, is not totally absent from the necrotic myocardium until this time. Ex vivo coronary perfusion of TTC solution as done here may theoretically not lead to thorough permeation of the dye through the previously hypoperfused myocardial region due to a "no-reflow" phenomenon. This possibility was countered by placing the heart slices in a TTC bath after photography and re-photographing. A shift of the border unstained/stained myocardium after this second staining step was not observed, confirming the acceptability of the whole heart perfusion method. Myocardial regions affected by the no reflow phenomenon would undoubtebly not remain viable in any case.

4.1.2 Thrombolytic efficacy of r-scu-PA ± taprostene

It is possible to infer that the coronary thrombi formed by the method used are resistant to spontaneous breakdown as the clots remained occlusive over the experimental period in both LAD and LCX groups in the vehicle treated control groups.

That r-scu-PA infusion can successfully recanalise an experimentally induced coronary thrombus has been shown here, and by other authors. The rapidity and success rate of fibrinolytic recanalisation is undoubtedly dependent on various thrombus parameters including its composition, age, morphology, site and the haemostatic and haemodynamic environment. As many diverse methods of experimental coronary thrombogenesis have been described, the extent to which the resulting thrombi affect experimental thrombolytic outcome should be considered: After using an intraarterial copper coil to precipitate LAD thrombosis, Collen's group induced thrombolysis in all 4 dogs with a 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ r-scu-PA infusion, the mean time required was 14 ± 3 min (±S.D.) (Van der Werf, Jang and Collen 1987).

Using Lucchesi's intraarterial anode thrombogenesis model, Söhngen (1988) required a greater r-scu-PA total dose (250 $\mu g \cdot kg^{-1}$ bolus plus 25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion for 30 min) to produce comparably rapid (16 ± 4 min ($\bar{x}$ ± S.E.M.)) and thorough (100 % of animals) recanalisation of the LCX. This apparent discrepancy might be explained by the proportionately greater contribution of platelets to arterial obstruction in the latter model (and in man) (Romson, Haack and Lucchesi 1980).

The efficacy of r-scu-PA in breaking down thrombin-induced coronary thrombi in dogs has not been reported previously to this author's knowledge. Collen's group however (Flameng et al. 1986) reported a recanalisation time of 21 ± 4 min ($\bar{x}$ ± S.D.) in a corresponding open-chest baboon model after infusing r-scu-PA at 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v..

Lijnen (Lijnen, De Wreede, Demarsin et al. 1984) examined the species variation in the susceptibility of plasma clots immersed in autologous plasma to lysis in vitro by E.coli-derived r-scu-PA. The following order of clot susceptibility to lysis was found: human > baboon > dog/rabbit. Extrapolating this observation to the present dog in vivo model a slower rate of recanalization (with the same thrombin thrombi and r-scu-PA dose) than in the baboon would be expected. The lysis rates were however similar (25 ± 5min dog LAD, 22±2 min dog LCX ($\bar{x}$ ± S.E.M.)). A possible reason for the greater than expected thrombolytic rate in dog studies is that in these experiments heparin was given together with r-scu-PA whereas in the baboon model it was given after coronary reperfusion. Heparin potentiates the conversion of n-sc-UPA to the more active 2-chain form in vitro, and enhances glu-plasminogen activation by the latter (Radzewski, Takada and Takada 1989; Lijnen and Collen 1986 a). Heparin may promote n-scu-PA thrombolysis in man (Gulba, Fischer, Reil et al. 1988).

The susceptibility of thrombin-induced coronary thrombi to in vivo lysis by r-scu-PA may be greater than that of electrically induced thrombi, but copper coil induced thrombi seem to be most susceptible to lysis. There are, however, other variables; the copper

coil model does not require thoracotomy, in contrast to the other two models, and surgery is widely known to imbalance the endogenous coagulation/thrombolytic system in favour of the former.

The concentration of acute phase inflammatory reactants including PAI-1 in thoracotomized animal models might possibly be more similar to the clinical concentrations after AMI than the closed chest model concentrations of reactants are.

In the current experiments exogenous thrombin will have activated both plasmatic and cellular haemostatic mechanisms, and residual thrombin activity could potentially inactivate a fraction of the r-scu-PA locally thereby delaying the lysis (Gurewich and Pannell 1987).

In the coronary thrombolysis experiments reported here the mean times to recanalization of the LAD and LCX arteries were very similar: 25 ± 5 min (LAD, n=4, $\bar{x}$ ± S.E.M.) and 22 ± 2 min (LCX, n=9, $\bar{x}$ ± S.E.M.) after starting the r-scu-PA infusions (infusion rate 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Taprostene coadminstration did not significantly increase the rate of restoration of LCX patency at the dose levels tested here.

After restoration of artery flow the flow probe was removed, as in the initial LAD experiments the flow probe often became displaced from the artery. No data are therefore available from these (LCX) experiments on the effect of coadminstration of the prostacyclin analogue taprostene with r-scuPA on the mainenance of artery patency after recanalization, and of the effect of taprostene coadministration on the attenuation of reflow rate after ischaemia due to microvascular perfusion failure.

At an arterial level, experimental and clinical thrombolysis is frequently followed by reocclusion. Prostacyclin infusion, at a hypotensive dose (400 $ng \cdot kg^{1} \cdot min^{-1}$) prevented coronary artery thrombosis in response to electrical injury of the intima (Romson, Haack, Abrams et al. 1981) and to severe arterial stenosis (Aiken, Gorman and Shebuski 1979).

Two anecdotal clinical reports in 1983 suggested that intracoronary prostacyclin infusion seemed to greatly increase the rate of recanalization occuring with streptokinase and urokinase therapy (Blasko, Berentey, Harsanyi et al. 1983; Uchida, Hanoi, Hasegawa et al. 1983). Another group provided limited clinical evidence of increased coronary recanalization by cotreatment of intracoronary streptokinase with PGE_1, a compound with similar actions to PGI_2 (Sharma, Wyeth, Gimenez et al. 1985). These observations were made under uncontrolled conditions and must be weighted accordingly. Schumacher et al. (1985) also demonstrated that intracoronary infusions of PGI_2 enhanced the reperfusion flow rate after streptokinase lysis of electrically induced LCX thrombi in dogs, and decreased the remaining thrombus mass, even though MABP was lowered by 35 mm Hg. These effects were attributable in part to concommitant heparin therapy, however. Prostaglandin E_1 (PGE_1) has recently been shown to increase the rate and extent of tPA-induced lysis of fibrin-rich venous thrombi in rabbits (Vaughan, Plavin, Schafer et al. 1989) and to promote urokinase-induced lysis of platelet-rich, but not platelet-free fibrin clots in vitro (Terres, Beythien, Kupper et al. 1989). Similar effects might be expected after PGI_2 or taprostene cotreatment with thrombolytic agents in vitro and in vivo.

Consequently great disappointment surrounded the results of a recent controlled clinical trial of iloprost cotreatment with tPA in 2 groups of 25 acute myocardial infarction patients.

Ninety minutes after starting the treatment, arteriographic patency was 60 % in the tPA group and 44 % in the iloprost plus tPA group ($p = 0.26$). The ventricular ejection fraction was increased at 7 days in the tPA group and decreased in the combination group. Iloprost coadministration (2 $ng \cdot kg^{-1} \cdot min^{-1}$ for 48 hours) markedly attenuated the fibrinolytic and fibrinogenolytic activity of tPA (Topol, Ellis, Califf et al. 1989). This negative effect of iloprost cotreatment on tPA thrombolysis was confirmed experimentally in a dog model of electrically induced LAD thrombosis (Nicolini, Mehta, Nichols et al. 1990). Here the frequency of successful reperfusion was similar between groups (≈ 65 % of animals recanalized) but iloprost coinfusion (4 µg/kg over 40 min) doubled the time to recanalization ($p < 0.02$) and dramatically reduced the duration of patency thereafter. Finally, the authors provided sound evidence that iloprost reduced circulating levels of tPA by 30% by enhancing hepatic flow and hence possibly tPA degradation. Although marked platelet inhibition ex vivo was found in the combination group this paradoxically did not prevent reocclusion. In the experiments reported in this thesis there was no evidence of an effect of taprostene coadministration, at the 2 sub-hypotensive doses, to either enhance or attenuate the thrombolytic effect (speed or success rate) of r-scuPA in this canine LCX model. That taprostene did not decrease the efficacy of r-scuPA in these experiments in a similar fashion to the iloprost/tPA thrombolysis experiments may be related to:

1. *The dose of taprostene may be too low for a marked interaction to occur*

2. *The fact that r-scuPA, in contrast to tPA, is not eliminated from the circulation entirely by a hepatic mechanism as shown by the fact that functional nephrectomy prolongs the plasma half life of scuPA, albeit to a lesser extent than does a functional hepatectomy (Stump, Kieckens, De Cock et al 1987).*

4.1.3 Haemodynamic parameters

MABP

The slow fall in mean blood pressure seen in both the LAD and LCX experiments over the 6 hour experimental period is probably accounted for by a combination of prolonged and repeated administration of pentobarbitone together with evolving AMI and by varying degrees of blood loss (diagram 15 and 24). The only significant fall in MABP due to drug treatment was in LAD thrombosed dogs treated with r-scu-PA. As this did not occur after r-scu-PA thrombolysis of the LCX (although the final quantity of r-scu-PA infused was 50% less in the latter) it could be concluded that this is not due to a direct systemic effect of r-scu-PA. Hypotension is possibly aggravated by the greater blood loss in the LAD model. The possibility of reperfusion induced hypotension, caused by more severe arrhythmogenesis in the LAD model was not examined. A theoretical explanation for moderate hypotension seen after systemic plasmin generation would be plasmin's effect of liberating the hypotensive mediator bradykinin from high molecular weight kininogen in plasma (Ver-

straete and Vermylen 1984).

Although a slight tendency was apparent, at the doses used, taprostene treatment did not significantly lower blood pressure (MABP) in r-scu-PA recanalized animals (LCX model). This is important as the left ventricular subendocardium, the most susceptible region to myocardial ischaemia, is perfused solely during ventricular diastole and the perfusion rate is dependent, apart from microvascular changes, on a) the pressure gradient aortic- root/left ventricle (during diastole) and b) the relative duration of diastole.

Thus any arterial vasodilator, given at the time of reperfusion, including taprostene at higher doses, might theoretically prolong subendocardial ischaemia by decreasing the perfusion pressure. This effect may be further aggravated when a remaining critical stenosis, a result of incomplete clot lysis, causes the maldistrubution of reflow blood to non-ischaemic myocardium.

Arteriodilator drugs may cause reflex tachycardia which greatly reduces the relative duration of diastole. Increasing heart rate from 65 to 200 min^{-1} decreases the absolute duration of systole and diastole by 47 % and 77 % respectively (Ganong, W.F. 1979). Increased sympathetic drive with attendant cardiac catecholamine release could cause further detriment by promoting myocyte necrosis, arrhythmogenic free fatty acid release, OFR production, platelet aggregability and by further imbalancing the local supply/ demand ratio of O_2. Taprostene treatment at both dose levels in the LCX experiments did not affect heart rate, or rate x pressure product, an estimate of myocardial energy requirement, and one of the 3 major determinants of experimental infarct size (Reimer, Jennings,

Cobb et al. 1985).

The premise is therefore that for any beneficial effect of taprostene to be seen here, it must possess a differential (lower) potency in its hypotensive effect compared to the "protective" effect and the dose must be adjusted accordingly.
In other words a bell-shaped, and not a sigmoid curve, may characterize taprostene's dose/myocardial-protection relationship. Two doses of taprostene were tested in this model to help in detecting this possible effect, and doses were employed that caused no marked hypotension.

L.V. peak pressure and contractility

The slow decrease in LVPP and +dP/dt over the experimental period was similar after both LAD and LCX continuous occlusion (diagrams 16, 17, 23, 15), but the smaller animal numbers in the LAD experiments resulted in greater variability. The causes of decline in peak pressure and contractility are no doubt the same as those causing the fall in MABP as LVPP and MABP are directly related. Considering the groups of animals that underwent acute thrombolysis of the LAD and LCX, there was evidence of an acute deleterious effect of r-scu-PA thrombolysis on contractility in LAD- but not LCX-animals. The selectivity of this effect precludes the possibility of a systemic action, or of a direct action of the thrombolytic agent on myocardial contractility. The acute decrease in contractility in the LAD recanalized animals is most probably a result of the far greater arrhythmic disturbances compared to the

LCX animals. In the dog the LAD perfuses the apical and anteroseptal myocardium, whereas the LCX perfuses a larger mass of lateral free wall and papillary muscle (Reimer and Jennings 1979), thus from a purely mechanical point of view a greater global function defect would be expected after LCX occlusion as a larger expanse of ischaemic free wall would undergo decreased segement shortening or even systolic bulging and paradoxical compliance. The LCX groups did not exhibit a greater contractile defect on occlusion compared to the LAD dogs as assessed by the two parameters, LVPP and +dP/dt. This might be accounted for by a compensatory increase in contractility in the respective non-ischaemic regions. For this reason the global pump function parameters LVPP and +dP/dt are relatively insensitive to any effect, positive or otherwise, of the treatments on the function of the ischaemic segment itself.

The rates and extents of wall thickening and segment shortening can be measured without ventriculography by experimental implantation of piezo crystal pairs, but the apparatus was not available. Lavallee et al. (1983) showed after 1 hour of LAD or LCX ligation that 3 to 4 weeks of reperfusion are required before preischaemic segmental contractile function is fully regained. With longer periods of ligation, 2 to 3 hours, only partial recovery is observed after equally long reperfusion. Prolonged periods of reperfusion (> 72 h) are required to reverse the segmental contractile defect caused by even brief (15 min) periods of coronary artery ligation (Kloner, DeBoer, Darsee et al. 1981). Protracted biochemical and ultrastructural changes are also observed including the depletion of high energy phosphate compounds and, over longer ischaemic periods, of their precursors. Slow de novo synthesis of these may therefore

explain the need for a prolonged recovery period (Reimer and Jennings 1981; DeBoer, Ingwall, Kloner et al. 1977). Prostacyclin and iloprost have however been shown to decrease the depletion rate of high energy phosphates and precursors, even after starting the infusion as late as 1 hour after canine LAD ligation (Pissarek et al. 1987).

As myocyte necrosis does not occur after 15 min periods of ischaemia, the term myocardial "stunning" was coined (Braunwald and Kloner 1982). Beneficial effects of prostacyclin analogue treatment on the recovery of ventricular function have been reported. Iloprost infusion started moments before releasing a 15 min LAD ligature, in open chest barbital anaesthetized dogs, increased the systolic segment shortening fraction over the following 3 hours compared to both vehicle and an equieffective dose of vasodilator drug. This effect of iloprost was attributed by the authors (Farber et al. 1988) in part to the inhibition of superoxide production by PMN's.

Oxygen free radical scavengers infused just before or together with coronary artery reperfusion have been shown in most studies (15 of 19 reviewed by Opie 1989) to reduce stunning. All negative results were found in studies with periods of preceding ischaemia longer than 60 min including one in which an experimental coronary artery thrombolysis model (with r-scuPA) was cotreated with superoxide dismutase (SOD) (Vanhaecke, van de Werf, Jang et al. 1988). In the LCX experiments reported here the lack of an beneficial effect of taprostene cotreatment on the postischaemic ventricular function recovery may be due to a combination of factors; the excessively

long preceeding period of ischaemia and inadequate convalescence period allowed, as well as the poor sensitivity of global function measurements to detect regional segment contractile characteristics. The significantly lower contractility in the high dose taprostene group requires some consideration (diagram 25) as this may be a detrimental effect, at least in the acute reperfusion phase. The group mean value was lower prior to ischaemia than in the other 3 groups, and lower contractility seen later may thus be an artefact. Another possibility is that taprostene, either alone or in conjunction with the barbiturate anaesthetic or fibrinolysis may have a direct cardiodepressant effect. This is unlikely as the effect was most apparent in the post-infusion phase. Alternatively if taprostene shares prostacylcin's and iloprost's effect of inhibiting the release of neural catecholamine release during the washout (reperfusion) phase as a consequence of membrane stabilization (Schrör et al. 1981), then decreased contractility may result from lower β_1 sympathomimetic stimulation. This suggestion is supported by the lower incidence of arrhythmic activity seen in this group during the identical time period (t = 3 to 6 hours) to which lower α_1-adrenoceptor stimulation could have contributed (Benfrey, Elfellah, Olgivie et al. 1984; Culling, Penny, Cunliffe et al. 1987). There are however sporadic unrelated reports of a deleterious effect of prostacyclin treatment on ventricular function when instituted concomitant with mechanical reperfusion. In a somewhat abstract ischaemia/reperfusion model, the isolated buffer perfused Langendorff rat heart, pretreatment with three drugs possessing the putative common action of cyclooxygenase inhibition caused a 35% to 70% recovery of contractile force on reperfusion compared to untreated

controls (11 % recovery). Re-adding pathophysiological concentrations (500 pg/ml) of PGI_2 to the reperfusate caused a reversal of the salutatory effects of each of the 3 agents as contractile force recovered to only 15 to 22 % of normal (Karmazyn 1986). A complete contradiction of these results was provided by Berti et al (1988) using a very similar rabbit heart Langendorff model: cyclooxygenase inhibition worsened the contractile dysfunction seen after reperfusion, and pretreatment with a compound which stimulates endothelial prostacyclin production, defibrotide, protected against the deleterious effects of the cyclooxygenase inhibitors. An obvious difference between these experiments is that in the former PGI_2 would have been lower during the ischaemic phase (in the former). More comparable are the results obtained after cotreatment with prostacyclin and mimetics during coronary thrombolysis in vivo. Schumacher et al (1985) found that PGI_2 administered with streptokinase (and heparin) caused the regional contractile force to recover transiently and acutely to ≈ 60% of the preischaemic value whereas without PGI_2 treatment the value was approximately 30%. In a clinical study with 25 AMI patients per group Topol et al (1989) showed that ilopost coadministration with tPA caused a reversal in the partial recovery of the ventricular ejection fraction after 7 days seen with tPA treatment alone. The improvement in regional wall motion after tPA thrombolysis was also significantly reduced by ilopost cotreatment. These effects may however have been more directly related to an effect of ilopost to decrease the fibrinolytic activity of tPA by enhancing its elimination therefore to shorten the time to a return of the thrombotic overbalance in blood than to a direct effect of ilopost on the post ischaemic myocardial function.

Evidence for this lies in the reportedly diminished duration and extent of fibrinolytic activity and a concommittant enhanced coronary reocclusion rate.

Although not examined here, taprostene as a PGI_2 mimetic may decrease oedema in the reperfusion region. Chemotactic agents LTB_4, complement C_{5a} and C_{3a} and others are released during infarction (Hartmann et al. 1977, Sasaki et al. 1988, Rossen et al. 1985) and can cause PMN-mediated local oedema. Prostacyclin at a subhypotensive dose (50 ng/kg/min, i.v.) attenuated oedema formation (rabbit skin model) induced by complement fragments and LTB_4 (Rampart and Williams 1986). Oedema-induced myocardial stiffness could, depending on the extent and spatial location of the area, either hinder ventricular contractile force development and hence decrease the +dP/dt value or decrease any paradoxical systolic bulging of the segment and hence increase the peak pressure and maximum rate of increase in ventricular pressure.

The observed inverse relationship between increasing taprostene doses (0 to 0.215 $\mu g \cdot kg^{-1} \cdot min^{-1}$) and the degree of myocardial necrosis, estimated both morphologically or enzymatically, negates the most obvious explanation of decreased contractility by taprostene - increased infarct size.

In vivo under non-ischaemic conditions i.v. PGI_2 infusion caused an initial increase in stroke volume, +dP/dt value and cardiac output in anaesthetized dogs (Chapple, Dusting, Hughes et al. 1980; Allan, Follenfant, Lidbury et al. 1985) and in conscious dogs (Dinerman, Mehta and Nichols 1988). In coronary artery disease patients PGI_2 infusion (5 to 10 $ng \cdot kg^{-1} \cdot min^{-1}$) caused an increase in ventricular

ejection fraction (Auinger, Virgolini, Weissel et al. 1989) and cardiac index (Firth, Winniford, Campbell et al. 1983). The apparent inotropic effect of PGI_2 in vivo, and hence possibly taprostene, is dose dependent as higher doses (1 $\mu g \cdot kg^{-1} min^{-1}$ PGI_2 over 10 min in open chest anaesthetized dog) caused a moderate rise followed by a marked fall in +dP/dt. Possible explanations include a vagal reflex resulting from the stimulation of prostaglandin receptors mostly in the inferoposterior myocardium (reviewed Hinze, 1987), or a decrease in +dP/dt as a result of afterload reduction. It is unlikely that the prolonged and delayed effect seen in the LCX model is related to the former because of the different temporal characteristics. The most plausible explanations for the apparent decrease in +dP/dt due to taprostene may therefore be as a result of afterload reduction, or of enhanced intercostal bleeding in the combination group due to an antiplatelet action.

HR and MABP x HR

Heart rates for LAD and LCX experiments are shown in tables 1 and 6 respectively. The apparent decrease in HR in the LAD group treated with r-scu-PA is undoubtebly a false positive effect as it started before commencing the r-scu-PA infusion. This artefact is attributed to the variability of group means due to small numbers per group in the LAD pilot experiments. No similar effect was seen in the LCX group undergoing r-scu-PA treatment compared to untreated controls. In the LCX experiments occlusion produced a small (< 10 %) decrease in the unnaturally high heart rates, but the other treatments r-scu-PA, alone and with taprostene, exerted

no marked effect, although in the last 2 hour period the mean value in the r-scu-PA alone group was marginally higher. Heart rate is an interesting variable insofar as both myocardial energy demand and supply, especially in the subendocardium, are unfavourably affected by tachycardia. Taprostene did not, under the conditions tested, affect the heart rates. The heart rate-pressure-product, an index of myocardial metabolic demand, was therefore directly related to the blood pressure (MABP), which was only very marginally decreased by the high dose of taprostene. In a multicentre standardized experiment employing an unconscious open-chest canine LCX model the rate-pressure-product was found not to contribute significantly to the size of the infarct (I/AaR) after 3 hours occlusion and 3 days reperfusion (correlation coefficient r = 0.26) (NHLBI study: Reimer, Jennings, Cobb et al. 1985). On these grounds it may be assumed that taprostene's effect here on infarction was unrelated to the slight central haemodynamic changes induced at the 2 dose levels tested. The high heart rates recorded here are characteristic for the open chest anaesthetized dog infarction model as the NHLBI comparative study further demonstrated: Mean HR: 158 versus 121 beats/min for anaesthetized and conscious dogs respectively.

Effects of r-scuPA ± taprostene on haemostatic parameters, plasminogen, fibrinogen and α_2 antiplasmin

Flameng et al (1986) induced thrombi with thrombin in open chested primates (baboons) and then showed with the same treatment regimen (r-scu-PA infusion-rate and source same as in the current study)

that minimal systemic depletion of the 3 haemostatic parameters fibrinogen, α_2-antiplasmin and plasminogen occurred. Few bleeding problems were observed and efficient thrombolysis was achieved. This was contrasted in the same experimental series, with the non-specific effects of two chain urokinase (tcu-PA) at doses required to produce comparable thrombolytic action because marked systemic depletion of the 3 haemostatic parameters occurred at equieffective doses. Söhngen (1988) also using r-scuPA from the same E. coli source found that similar infusion rates of r-scuPA, preceded however with a bolus, caused marked systemic degradation of fibrinogen and other haemostatic factors, which implied that systemic plasmin generation was occuring.

In the LAD series of experiments r-scuPA infusion produced significant decreases in fibrinogen (≈ 15% decrease), α_2 antiplasmin (≈ 30-40% decrease) and a moderate (≈ 20%) decrease in plasminogen levels in plasma (diagrams 18, 19, 20). The decreases in these plasma parameters were less in the LCX experiments after infusion of r-scuPA at the same rate (20 $\mu gkg^{-1}min^{-1}$) but for a 30 min instead of a 60 min duration (diagrams 30, 31, 32). Fibrinogen levels were unaffected, plasminogen levels decreased marginally and antiplasmin activity was decreased by ≈ 20%. One of the two obvious variables between the two series of experiments, the total dose of r-scuPA infused, might account for the greater decrease in the haemostatic parameter levels in the LAD experiments although it is difficult to imagine that a higher peak or steady state level would result form the different durations of infusion because of the short plasma half life ($t_{1/2}\alpha$) of 7 to 9 min of most forms of scuPA

(Stump, Kieckens, De Cock et al 1987). It may therefore be that the degree of systemic degradation of these parameters is a function of both the peak level of scuPA and the length of the exposure period. This supposition cannot be confirmed with this data partly because the other variable, the site of the coronary thrombosis, was associated with a marked difference in the tendency to bleed as discussed above. In LAD animals, bleeding was enhanced through fine artery branches after thrombolysis, and saline infusions were given to replace the blood. These may have caused haemodilution resulting in an artefactual decrease in the plasma levels of the haemostatic parameters in the LAD experiments.

In the PRIMI clinical trial infusion of r-scuPA (20 mg bolus, 60 mg infusion over 1 hour) at an average dose rate of 15 to 20 $\mu gkg^{-1}min^{-1}$ (converted) caused a fall in the plasma fibrinogen levels, as estimated by the Clauss assay, from a mean pretreatment value of 2.5 g/l to 0.44 g/l at the end of the infusion (PRIMI Trial Study Group 1989). These results seemed to show that the fibrin specificity of r-scuPA was limited and that systemic plasmin generation caused extensive fibrinogen degradation. A very recent study, comparing four different assays of fibrinogen using samples from the above clinical study showed that the Clauss assay underestimates the level of fibrinogen after thrombolysis due to negative interference of fibrinogen degradation products on the assay's clotting-time end point. Furthermore using a dual ELISA (enzyme linked immuno assay) which detects only fibrinogen molecules with at least one intact α chain it was shown that the levels of fibrinogen in the PRIMI trial were 60 % of pretreatment value 2 hours

after thrombolysis, whereas the Clauss assay produced results suggesting that the levels were 14% of pretreatment values at the same time (Hoffmann, Vijgen, Niewenhuizen 1990). Critical sagacity is hence of paramount importance in the interpretation of apparent changes in fibrinogen levels, as measured by the Clauss assay, due to fibrinolytic therapy.

The effects of r-scuPA infusion in the LAD and LCX models on the other two haemostatic parameters, plasminogen and AP are most likely directly related to each other. As plasminogen is present in plasma (in man) at a concentration of 2 µM and α_2 antiplasmin at 1 µM (Bachmann 1987), activation by r-scuPA of 10 % of the baseline levels of plasminogen would cause a decrease in AP activity of 20 %, as a result of 1:1 stoichiometric molecular complexation and mutual inactivation. This theory is borne out to a fair degree of accuracy in the results obtained here. The fact that rapid and efficient clot lysis occurs with a dose of r-scuPA that does not exhibit prerequisite depletion of plasma antiplasmin activity lends further credence to the various hypothetical models of fibrin specific action of r-scuPA.

Considering the effect of taprostene coinfusion on r-scuPA induced changes in the haemostatic parameter levels it must be noted that for plasminogen, and to a greater degree AP, high dose taprostene seemed to reduce these indirect estimates of the degree of plasmin generation, at least in plasma.

4.1.4 Dog infarct size/discussion

Thrombolytic recanalization of the LAD artery after a mean duration of occlusion of almost 2 hours resulted in a 80% reduction in the 6 hour relative infarct size compared to continuous thrombolytic occlusion or ligation (Table 3, diagram 22). Thrombolytic recanalization of the LCX artery after an equally long duration of thrombotic occlusion and otherwise identical conditions resulted in a 47% reduction, compared to the 6 hour thrombotic occlusion controls (Diagram 28, table 7). Hence thrombolytic reperfusion of the antero-apical myocardium supplied by the LAD would appear to be more rewarding in terms of acute myocardial salvage than reperfusion of the inferior myocardium and papillary muscle supplied by the LCX in the dog. Other anatomic variables detract from the cogency of this conclusion however. The size of the anatomic risk area (AaR/LV), although quite reproducible in the LCX experiments (table 7, diagram 28) was markedly smaller and more variable in the LAD experiments (table 3, diagram 22) and as discussed above smaller areas at risk have been shown to have higher specific myocardial perfusion rates via coronary colaterals (Gumm et al. 1988). Intrapolating data obtained by Reimer and Jennings (1979) after LCX ligation and reperfusion in dogs, sacrificed after 4 days, suggest a potential for 40% reduction in

infarct size after reperfusion following 2 hours ischaemia compared with permanent occlusion. This value is similar to the one found in the present LCX study with thrombolytic recanalization.

There is some evidence that release of a coronary artery ligature after a given period of occlusion does not necessarily lead to the same degree of infarction as thrombolytic recanalization does after the same period of preceding ischaemia (Darius, Yanagisawa, Brezinski et al. 1986). These authors reported that tPA infusion concomitant with the release of a 2 hour LAD ligature reduced the 6 hour relative infarct size from 47% to 30% ($p < 0.02$) as well as attenuating the release of myocardial CK activity compared to vehicle infused controls. Haemodynamic and vasoactive effects of tPA were dismissed by the authors as possible explanations and although the potential fibrinolytic action of tPA to dissolve microthrombi and enhance palliative reperfusion could not be excluded, the possibility of a "cytoprotective" effect was speculated on by this group. Another group showed in Langendorff perfused rabbit hearts that crystalline perfusates containing streptokinase (150 U/ml) partially reversed the detrimental effects of an electric current on myocardial vascular and contractile function. This effect was attributed to oxygen free radical scavenging by the thrombolytic agent (Mickelson, Simpson, Jackson et al. 1988). The same group later showed that intracoronary streptokinase infusion maintained reperfusion coronary blood flow through a critical stenosis after 90 minutes of LCX ligation in vivo. Infarct size was not altered compared to vehicle controls, nor was in vitro platelet aggregation (Mickelson, Simpson and Lucchesi 1989). These results taken together suggest that at least

certain thrombolytic agents may exert effects on the evolution of infarction independent from restoring artery patency. Other workers have been unable to repeat the result using an in vivo canine LAD model with tPA infusion during the reperfusion phase however (Kloner, Alker, Campbell et al. 1989).

Other reasons why thrombolytic recanalization cannot be directly compared with the release of a coronary ligature are

(a) the effect of a possible rest thrombus in the former causing stenotic flow conditions which have a detrimental effect on the terminal infarct size and ventricular function, albeit in animals not treated with fibrinolytic agents (Schmidt, Varghese, Bloom et al. 1986; Lefkowitz, Pace, Gallagher et al. 1988).

(b) the effect of fibrinogenolysis and FDP generation after thrombolysis on the rheological and haemostatic characteristics of blood, especially at a microvascular level, discussed later.

(c) the effect of plasmin generation on protease sensitive inflammatory mediator systems including the kinin and complement systems (Bennet, Yawn, Migliore et al. 1987; Bachmann 1987)

(d) the effect of at least some plasminogen activators on platelet activity (reviewed Coller 1990).

The results of present LAD and LCX experiments confirm that early r-scuPA infusion interrupts the progression of myocardial necrosis after coronary artery thrombotic occlusion by restoring myocardial perfusion secondary to thrombolytic recanalization.

Plasma creatine kinase (CK) activity was recorded in these

thrombolysis experiments as an index of the extent of myocardial necrosis. As mentioned in the "methods" section no assay is available for the relatively myocardium specific CK-MB isoenzyme fraction in dogs, but in these experiments a standardized experimental protocol eliminated a bias effect of other possible sources of CK activity, for instance after intercostal muscle incision. Furthermore in contrast with CK enzyme release by the myocardium, skeletal muscle incisions would tend to bleed externally, avoiding recirculation of the enzyme activity.

Confirming this effect, CK plasma activity between thoracotomy and the onset of myocardial ischaemia was normal (≈ 70 U/l) in all animals.

The rapid increase in plasma CK activity in animals undergoing coronary artery reperfusion, compared to animals with continuous myocardial ischaemia, as observed here (diagrams 21 and 33) has been reported in animal models with coronary artery ligation with and without reperfusion (Vatner, Baig, Mander et al. 1978) and in hypoperfused isolated rat hearts (Mohanlal, Moeve, Zoet et al. 1988). The accelerated rise in CK activity associated with myocardial reperfusion is speculatively attributed to "reperfusion injury" (Bresnahan, Roberts, Shell et al. 1974) or to the rapid "washout" of the enzyme from myocardial regions undergoing accelerated necrosis after reperfusion (Van der Laarse, van der Wall, van den Pol et al. 1988; De Vries, Jaffe, Geltman et al. 1989). A rapid rise in plasma CK activity in patients is now generally accepted as an inidicator of successful coronary artery recanalization after thrombolytic therapy (Garabedian, Gold, Yascuda et al. 1988; Lewis, Ganz, Laramee et al. 1988).

De Vries et al. (1989) also demonstrated that the rate of increase in plasma CK activity corelated closely with the infarct size when CK release was measured during the first 60 minutes after reperfusion (r = 0.92). This is obviously only valid after reperfusion, and considering the animals undergoing reperfusion in the present LCX study a significant reduction in the CK plasma activity was seen after high dose taprostene cotreatment compared to r-scuPA alone, implying a reduction in infarct size. Using De Vries' method, calculation of the rate of increase in CK activity between t = 2 and t = 3 h (t = 2 h roughly corresponds to the onset of reperfusion) yielded the following values - group II (r-scuPA alone): 440 Uxh/l, group III (low dose taprostene + r-scuPA): 285 Uxh/l group IV (high dose taprostene + r-scuPA): 115 Uxh/l. Therefore, on the basis of the rate of increase of plasma CK activity over the first 60 min of reperfusion being directly proportional to infarct size, low and high dose taprostene cotreatment with r-scuPA, starting late in the ischaemic phase, reduced the enzymatic estimate of infarct size by 35% and 65% respectively compared to r-scuPA treatment above. This is only moderately comparable with the planimetric estimates of infarct size after TTC staining where relative reductions in infarct size of 16% and 32% were recorded in the low and high dose taprostene cotreatment groups compared to the group treated with r-scuPA alone. Greater absolute increases in mean plasma CK activity 4 hours after LAD reperfusion (group C, diagram 21) were recorded compared with the equivalent LCX reperfused group (group II, diagram 33) despite the smaller infarcts in the former group. This might be explained by the smaller anatomic risk area in the former (Aar/LV) which enables the CK released after infarction to be eluted more rapidly.

Therefore in order to compare the enzymatic estimate of infarct size between 2 groups with markedly different anatomic risk areas (AaR/LV: LAD ≈ 20%, LCX ≈ 40%) the area under the plasma CK activity/time curve may be more appropriate using an extended observation period (≈ 24 h).

The mechanism by which taprostene cotreatment, started relatively late in the ischaemic phase, can cause a trend to a further reduction in infarct sizes (enzymatic and planimetric estimates) compared to r-scuPA alone is a point of prime interest in this thesis. In virutally all experiments in which it was shown that prostacyclin and mimetics could exert an antinecrotic effect after myocardial ischaemia with and without reperfusion, first exposure of the myocardium to the compounds occurred early during the ischaemic phase or even prior to ischaemia.

Considering the mechanisms suggested from these experiments by which prostacyclin and mimetics have reduced terminal infarct size, a number of these possible mechanisms can be considered in relation to the current experiments.
A major contributary effect of taprostene here on the myocardial energy demand/supply balance during the ischaemic period can be excluded with a moderate degree of surety. Taprostene infusion started after 90 minutes of ischaemia, and approximately 25 minutes later thrombolytic recanalization was achieved (Table 7), therefore the taprostene "exposure" period was only during the last fifth of the ischaemic period. As no changes in any of the haemodynamic parameters occurred during this period it can at least be stated that the effect of taprostene cotreatment to reduce the infarct sizes

was not related to a reduction in myocardial energy demand during the ischaemic phase. An effect of taprostene on the energy supply to the ischaemic region during ischaemia cannot be rejected in these experiments as the collateral blood flow to this region was not measured.

Jugdutt et al. (1981) reported in a continuous LCX occlusion model in conscious dogs that PGI_2 reduced the infarct size by increasing collateral flow to the ischaemic region. However the dose of PGI_2, starting 5 minutes after ischaemia, produced hypotension and relative infarct size reduction by PGI_2 was also seen in dogs with low levels of collateral flow. Other groups have since shown a reduction in infarct size by PGI_2 and iloprost in the absence of an effect on the local collateral flow rates and haemodynamic parameters (Melin and Becker 1983; Chiariello, Golino, Cappelli-Bigazzi et al. 1988). Furthermore the ischaemic myocardium becomes unresponsive to the effects of prostacyclin mimetics on the local distribution of myocardial blood flow (Smith, Gallenkämper, Beckmann et al. 1984; Simpson et al. 1987; Farber et al. 1988) which precludes the remote possibility of taprostene increasing the local blood flow during ischaemia.

Following the restoration of LCX artery patency however two effects of taprostene coadministration could potentially reduce the infarct size at 6 hours. By maintaining myocardial perfusion after thrombolysis, at any level from preventing coronary artery reocclusion to decreasing the "no-reflow" effect at a capillary level or by decreasing the postischaemic energy demand. The lower ventricular contractility associated with high dose taprostene cotreatment

group (diagram 25) would not account for the reduction in infarct size in this group (diagram 28) as it occurred late in the reperfusion phase. Myocardial cells "condemned" to die during the ischaemia phase but still with an intact cell membrane rapidly become necrotic with the onset of reperfusion (Hearse and Yellon 1984). Maintenance of myocardial perfusion after thrombolysis either by preventing the formation of occlusive platelet aggregates or by reducing the no-reflow phenomenon could prevent extension of the period of ischaemia despite successful thrombolysis. As demonstrated here in the rabbit carotid artery stenosis experiments (diagrams 35, 36, 37) cotreatment of taprostene with r-scu-PA effectively abolishes acute arterial thrombosis. In the LCX model coronary artery flow was not recorded after recanalization occurred and it cannot be excluded that reinfarction occurred in the untreated animals (group II) however this is unlikely as a continuous washout of CK from the myocardium was recorded over the experimental period. The ability of prostacyclin mimetics to reduce experimental infarction can be dissociated from the in vivo antiplatelet effects (Schrör et al. 1982), suggesting that other effects are involved.

Extensive evidence exists for a major role of PMN and oxygen-free radicals in tissue injury after ischaemia and reperfusion (review: Lucchesi and Mullane 1986). The cardioprotective effects of prostacyclin and mimetics have increasingly been attributed at least in part to an inhibition of neutrophil accumulation, activation and oxygen-free radical production (Farber et al. 1988; Simpson et al. 1987; Thiemermann et al. 1984). A number of important differences distinguish these studies from the experiments in the present study

however.

The first difference is that the time of initial exposure of the prostacyclin or mimetic was shortly after or even before the onset of ischaemia. As this bears little relevance to a clinical situation a protocol was employed here in which the taprostene infusions were started together with r-scu-PA, after 90 minutes of ischaemia. The second difference is that no study to date has compared experimental infarct sizes after a prostacyclin mimetic was infused together with a fibrinolytic agent.

Considering the first difference, one study compared the effect on contractility of iloprost infusions when started at the onset of or end of a 15 min period of LAD ligation in open chest pentobarbitone anaesthetized dogs (Farber et al. 1988). In this study it was shown that iloprost treatment starting immediately prior to reperfusion exerted a beneficial effect on the recovery of contractile function of the ischaemic segment compared to controls, although earlier iloprost treatment resulted in greater recovery. The authors rejected an effect of iloprost on the xanthine oxidase-free radical generating system and attributed its effect to a reduction in the superoxide radical production by iloprost in response to chemotactic stimulation. Infarction would not have resulted from such brief periods of ischaemia however. No studies have examined a potential in vivo interaction: PMN function/thrombolysis/myocardial infarction, but an effect of thrombolysis to enhance PMN mediated reperfusion injury must be considered from a theoretical basis; fibrin degradation products (FDP), produced in high concentrations during thrombolysis, are chemotactic for PMN (Becker 1983) and may enhance their accumulation and activation in the reperfused myocardium. If

FDP do contribute significantly to this effect then fibrin specific thrombolytic agents may in fact have a more detrimental effect on the myocardium than unspecific agents as they would produce a much higher chemotactic gradient between the ischaemic and normal myocardium. Additionally some thrombolytic agents enhance thrombin activity (Seitz 1988) which leads to the liberation of factors in plasma which induce neutrophil aggregation, adherence to the endothelium and superoxide production (Lo, Lai, Cooper et al. 1988). Also protease activity synergistically enhances the toxicity of oxygen-free radicals (Varani, Ginsburg, Schuger et al. 1989). In contrast, t-PA and plasmin have been reported to decrease PMN activation in vitro (Mehta, Mehta, Lawson et al. 1989). If thrombolytic therapy with r-scu-PA does enhance PMN-mediated cytotoxicity then the ability of taprostene to potently inhibit PMN activation (Stahlberg et al. 1988) would represent a further mechanism in which taprostene cotreatment led to smaller relative infarct sizes in the present experiments.

To summarize the effects of taprostene cotreatment with r-scu-PA on the infarct size in this model it has been shown in this study that taprostene cotherapy reduces the infarcts size compared to r-scu-PA alone. This was demonstrated with 2 independent indices of myocardial necrosis, TTC-staining of viable tissue and by the release of enzyme activity into plasma after cell necrosis. The purpose of this study was to determine whether taprostene cotreatment with r-scu-PA at such a late phase in ischaemia could exert further anti-necrotic effects, which it did.

The study was not designed to enable the protective mechanimsms to be elucidated, but an effect of taprostene on the myocardial energy demand/supply balance can be largely discounted here.

4.1.5 Arrhythmias/Dog

A marked increase in arrhythmia at the infarct reperfusion is commonly reported in coronary artery ligation models (review Sheridan 1987), and after restoration of coronary artery patency by clinical thrombolysis (Pop, Erbel, Treese et al. 1987; Goldberg, Greenspan, Urban et al. 1983). The intensity of reperfusion arrhythmia is related to the severity of antecedent ischaemia, including residual collateral flow and also to the relative mass of ischaemic myocardium and the heart rate (Bolli and Patel 1988). The mechanisms involved have eluded comprehensive evaluation (Manning and Hearse 1984) but recently the involvement of oxygen free radicals has attracted much attention (Manning 1988).

Prostacyclin infusion during myocardial ischaemia decreases the intensity of arrhythmia at subhypotensive doses when there is no tachycardia (Johnston et al 1983, Starnes et al 1982) but higher doses are arrhythmogenic (Au et al. 1979). Treatment with prostacyclin during ischaemia also reduced the intensity of reperfusion arrhythmias (Coker and Parratt 1983). Three possible actions of taprostene may account for the reduction in reperfusion arrhythmia seen here (diagram 29). They are by inhibiting the accumulation of PMN and their release of oxygen free radicals and other noxious products in the reperfused myocardium (review by Lucchesi and Mullane 1986), by a membrane stabilizing action similar to propranolol (Hieda et al. 1988) which may decrease the breakdown of phosphatidyl choline and ethanolamine (Darius et al 1987) with the liberation of arrhythmogenic compounds (Corr et al 1984), or by decreasing 2 determinants of reperfusion arrhythmias, the severity of ischaemia prior to reperfusion or the infarct size itself.

The antiarrhythmic effect of taprostene seen here was not dependent on treatment early during ischaemia, and is probably not dependent on haemodynamic effects at these doses. Diverse vasodilator drugs did not affect the occurence of ventricular arrhythmias and fibrillation after LAD occlusion and reperfusion in pentobarbitone anaesthetized open chest dogs (Elfellah and Ogilvie 1985), which would suggest that pre- and after-load reduction does not affect the occurence of reperfusion arrhythmia.

The involvement of PMN in reperfusion arrhythmia can be demonstrated by leucocyte depletion which reduced the occurence of reperfusion ventricular fibrillation from 60% to 10% of dogs (Engler 1987). Also using multiple linear regression analysis of baseline parameters from myocardial infarction patients on admission it was found that both the initial and peak PMN counts were the most important predictors of acute ventricular fibrillation (Maisel, Gilpin, Le Winter et al. 1985). Other drugs including nafazatron and BW755C reduced the incidence of reperfusion arrhythmias concurrently with a reduction of postischaemic PMN accumulation similar to that caused by prostacyclin (Simpson et al 1987). Thus assuming the arrhythmic activity is related to PMN activity the antiplatelet action of taprostene may contribute to the antiarrhythmic activity as the presence of platelets increases PMN activity (Dinerman, Metha, Lawson et al. 1988). Furthermore some thrombolytic agents may decrease neutrophil function (Mehta, Mehta, Lawson et al. 1989).

Although the antiplatelet action of some prostacyclin analogues is not required for an antiischaemic effect to be seen (Schrör et al.

1982) and platelet depletion does not affect the infarct size after ischaemia (Mullane et al. 1985) platelets may contribute to arrhythmias via thromboxane production (Parratt and Coker 1985). In myocardial infarction in man platelet activity increases greatly and marked resistance to exogenous prostacyclin is seen (Müller et al. 1985). The compensatory increase in vascular production of PGI_2 in ischaemia is decreased after (thrombolytic) reperfusion (with tPA) (Kerins et al. 1989) which is indicative of reperfusion injury to the endothelium. Therefore this provides a further rationale for supportive exogenous prostacyclin treatment during thrombolysis. The antineutrophil effect of taprostene (Stahlberg et al. 1988) may have a further indirect effect on platelets as neutrophils have been shown to regulate the accumulation of platelets in the reperfusion myocardium (Mullane 1989).

The dose-dependent reduction in arrhythmic activity by taprostene cotreatment with r-scuPA is probably connected with the dose-related decrease in infarct sizes (enzymatic and morphological indices) either directly, or by an affect of taprostene to reduce (or postpone) some disruptive mechanism in the reperfused myocardium. Current knowledge would suggest that this mechanism is the deleterious effect caused by neutrophils accumulating in the myocardium on reperfusion. As a final comment, it was hoped that the accumulation of PMN could be compared between groups here. After establishing the myeloperoxidase (MPO) assay which has been employed to quantify PMN presence (Williams, Paterson, Eatkins et al. 1983, Bradley, Priebat, Christensen et al. 1982) it was found that oxidized and reduced TTC present in the samples reacted differently in the MPO assay and the assay could not be applied in

these studies. Furthermore as haemoproteins also interfere with the MPO assay (Burkhardt and Tideman 1981) an artifact scenario is imaginable where reperfusion haemorrhagia is reduced by a treatment without an effect on PMN accumulation and MPO activity is paradoxically elevated.

4.2 Rabbit experiments/discussion

Early reocclusion, that is during or shortly after the infusion of thrombolytic agent is one of the factors limiting the success of early thrombolysis: on average 5% to 20%, but even up to 45% of successfully treated patients reocclude at this stage (Heras, Chesebro, Thompson et al 1989). The risk of reocclusion after successful thrombolysis may, (Harrison, Ferguson, Collins et al 1984) or may not, (Ellis, Topol, George et al 1989) be directly linked to the degree of persisting stenosis. Furthermore, intermittent occlusion and reopening of the coronary artery during fibrinolysis (and beforehand) has been demonstrated electrocardiographically in AMI-patients (Hackett, Davies, Chierchia et al 1987) suggesting the instability of at least some early reocclusions. The pathiophysiological mechanisms operating here may therefore be the same as those causing cyclic arterial flow variations (CFR) in animal models. The initial studies on CFR were done by Folts and coworkers (Folts, Gallagher and Rowe 1982) on externally stenosed dog LCX arteries. A 60-80 % reduction in luminal diameter abolished the postischaemic hyperaemic response and caused CFR, eventually leading to LCX occlusion.

In the Folts Model, prostacyclin (Aiken, Gorman and Shebuski 1979), aspirin (Folts, Crowell and Rowe 1976), α_2 and/or $5HT_2$ receptor blockade (Bush, Campbell, Kern et al 1984), TxA_2 synthetase inhibition (Bush, Campbell, Buja et al 1984) and TxA_2/PGH_2 receptor blockade (Ashton, Schmitz, Campbell et al 1986) all inhibited the CFR. This, in addition to angiographic and microscopic evidence, suggests an inappropriate but central role of platelets in

obstructing the arterial flow. In addition, ancrod, an extract of viper venom which cleaves fibrinogen rendering it unclottable, was shown to reduce the frequency of CFR, albeit to a lesser degree, in a similar model (Apprill, Ashton, Guerrero et al 1987). This suggests that fibrin(ogen) stabilizes platelet aggregates involved in producing CFR. It may therefore be reasonable to expect, on this basis alone, that any possible advantage of "fibrin-specific" thrombolytic agents is offset by an inherently greater tendency to reocclude and that antiplatelet drugs may make a major contribution to the maintenance of artery patency as a thrombolytic adjunct, especially in situations where thrombolytic therapy causes platelet hyperactivity.

A model was required to examine the separate and combined effects of taprostene and r-scu-PA on simulated arterial thrombosis and therefore, indirectly, the tendency to reocclude after thrombolysis.
These experiments could not have been done in the preceeding dog study in parallel, for instance on another coronary artery, due to the possible effects on redistribution of coronary blood-flow, global ventricular function and resulting extent of infarction in the latter.

A first series of attempts not reported in this thesis to find a reproducible model of arterial thrombogenesis involved Cotton thread, soaked in thrombin, inserted through the carotid artery lumen (axis) over fixed distances using a surgical needle. This method had three major drawbacks: occlusion was very rapid, complete, and virtually irreversible; the arterial segment also

underwent intense spasm possibly due to mechanical injury and/or the effect of thrombin on platelets and endothelium to elicit the release of vasoactive agents, and lastly treatment with r-scu-PA led to profuse (preferential) bleeding through the puncture holes. This model would also not allow discrimination between the potential antiplatelet and any antispasmic effect of taprostene. In conclusion this model was flawed with many unpracticalities and was therefore rejected.

The rationale for the final model used are as follows:
Implantation of polythene tubing of a previously established optimum length and internal diameter enabled standardization of the stenosis and to a limited extent the shear rate. The rigidity eliminated local vasomotor effects.

In these experiments the test substances were infused for 10 minutes prior to the introduction of the stenosis. This was necessary because in the rabbit, as between the aortic arch and the first bifurcation to the facial artery the carotid artery runs for ≈ 5 cm. Distal thrombosis in this column would preclude access of the drugs to the thrombus if given later, and any recanalisation would most likely have occured spontaneously, at least initially.

Very little data is available on the effect of r-scu-PA on platelet function in vivo or in vitro, and extrapolating data from Streptokinase or tPA may be inappropriate. Apparently conflicting observations of enhanced and decreased platelet aggregation after exposure to tPA and SK.

In the present experiments r-scu-PA significantly increased the mean interval flow rate through the stenosis at the end of the infusion period (30 - 60 min) and later on (90 - 120 min after the end of the infusion). In rabbits and in primates the rate of disappearance of r-scu-PA antigen, ^{125}I-label and fibrinolytic (ECLT) activity was shown previously to be extremely rapid ($t_{1/2\alpha}$ 3 - 6 min) (Collen, De Cock and Lijnen 1984). Although the hepatic clearance mode shows extreme dominance it cannot be excluded that a small quantity of scu-PA resists elimination for example by binding to endothelium or blood elements as recovery of label was not complete in these studies. The results of these ex vivo lysis rate studies showing rapid decay of lytic activity after stopping scu-PA infusion may be queried for two reasons: scu-PA is partially and variably precipitated in the ECLT fraction at pH 5.8 (Gurewich, Pannell, Louie et al 1984) and thrombin, added to form the ex vivo clots, degrades scu-PA.

These studies provide reasonable evidence that a form of functional cooperation exists between taprostene and r-scu-PA in preventing arterial thrombotic occlusion. The presumed separate effects of the two compounds, that is antiplatelet and fibrinolytic effects respectively, may play major roles in this cooperation, but there is also evidence in other studies of reciprocity, i.e. profibrinolytic action of prostacyclin (Winther, Snorrason, Knudsen et al 1987) and of an antiplatelet effect of fibrinolytic agents (review Coller 1990). In the case of fibrinolytic agents this is reportedly related to the formation of fibrin degradation prducts (FDP) (Kowalski, Kopec and Wegrzynowicz 1964) or to the proteolytic

degradation by plasmin of platelet aggregation cofactors (fibrinogen, clotting factors, platelet glycoprotein receptors. As no change in plasma fibrinogen levels was observed, this study dissociates the ability of r-scu-PA to prevent arterial occlusion, for the duration of the infusion, from an effect of producing systemic fibrinogen degradation. Furthermore the Clauss fibrinogen assay is sensitive to FDP presence in the plasma samples (Garbedian, Gold, Leinbach et al 1988). So another conclusion arising from the experiments is that the effect seen with r-scu-PA on arterial patency is probably not dependent on the generation of high concentrations of FDP and their subsequent effects on haemostasis. Probably of little relevance here, but an intriguing observation nevertheless, is that certain FDP cause endogenous prostacyclin release (Mehta, Nichols and Saldeen 1989).

Considering physical factors which would variably affect stenotic flow and thrombosis, as neither r-scu-PA nor taprostene affected the arterial blood pressure here, the flow rate would not have been directly affected. Hence, the shear rate, one determinant of the platelet deposition rate (Badimon, Benedict, Todd et al. 1987), would not be directly affected by the compounds.

The determination of plasma fibrinogen in this carotid flow study was necessary for another reason: for Newtonian fluids including plasma the flow rate is inversely proportional to viscosity. Fibrinogen, an elongated, large molecule is the major determinant of plasma viscosity; at 4 % w/w it accounts for the 30 % increase in viscosity beyond that of water alone and doubling of the plasma fibrinogen level increases its viscosity by 20 % (Rampling 1988).

Changes in plasma fibrinogen levels would therefore affect the stenotic flow rate. No changes in fibrinogen levels were, however, detected in any of the groups and therefore the possibility of this effect, especially after fibrinolytic treatment,may be rejected here. Furthermore the relatively rapid return of the tendency to occlude after cessation of the r-scu-PA infusion points to an ongoing effect and not to a marked depletion of the circulating pool of fibrinogen or clotting factors, or an effect on platelet count, because their low physiological replacement rates would result in a prolonged antiocclusive effect. This effect cannot be fully excluded here however as evidence exists of rapid repletion of fibrinogen after fibrinogenolysis, possibly by release of reserve pools.

The viscosity of whole blood is highly dependent on the haematocrit, which was not examined in these experiments, but any changes caused by blood sampling and haemodilution by the infusions were constant between all groups. Thrombolytic agents may lower the platelet count to a moderate extent starting shortly after infusion, and as this potential variable was not examined in these studies it cannot be excluded, although it is unlikely to have contributed much to the observed effect of r-scu-PA.

The frequency of cyclic flow variations in the r-scu-PA treated group was much higher than in the vehicle treated group. The obvious conclusion is that in the vehicle group occlusion was less reversible. Conversely, the frequent spontaneous increases in stenotic flow rate in the r-scu-PA group may represent variable combination of 3 factors:

a) ongoing local fibrinolysis with degradation of the fibrin net binding the platelet aggregates together.

b) reduced tensile strength of clots formed from fibrinogen previously exposed to limited proteolysis (Hirsch, Fletcher and Sherry 1986)

c) diminished stability of the interplatelet binding.

Of uncertain commonality is the effect of some thrombolytic agents to activate platelets, which might aid stenosis occlusion. (Kerins, Roy, Fitzgerald et al. 1989; Udvardy, Harsfalvi, Boda et al. 1990) This effect is yet to be examined two chain (tc) or single chain (sc) uPA, but a comparison with streptokinase (SK) here in this model would have been inappropriate as SK is a poor activator of lapine plasminogen (Reddy 1988).

The intentional omission of heparin in these experiments was for 2 reasons: Heparin treatment has been reported both to prevent, (Benedict, Todd, Sheng et al. 1988) and not to affect (Folts et al 1982) thrombosis resulting from experimental artery stenoses, and probably depends on the severity of stenosis. Heparin may enhance platelet aggregation (Ljungberg, Bevering, Egberg et al. 1988) or augment experimental thrombolysis (with SK) (Schumacher, Lee and Lucchesi 1985) thus treatment of the rabbits with heparin would have inextricably complicated any interaction between r-scu-PA and taprostene.

A number of important differences exist between the Folts' model (canine coronary artery stenosis) and this one, apart from the species difference: The site of the stenosis is important from at least two aspects, firstly the pressure/flow/time relationship in coronary arteries is different to that in peripheral arteries.
The second difference is the effect of myocardial ischaemia, resulting from severe artery stenosis and occlusion, on local and systemic haemostasis, in particular of enhancing platelet function (see discussion canine thrombolysis model).
Furthermore in a more traumatic open chest model haemostatic and sympathetic activation may dominate, with accompanying effects on haemodynamic variable and platelet aggregation.

In this model the choice of anaesthetic agent, pentobarbitone was a compromise. Pentobarbitone (i.v.) seemed to have a narrow therapeutic index in the rabbit and also seemed to cause a transient increase in cerebral blood flow when given rapidly. For this reason it was diluted and given slowly as required at about 20 minute intervals.

4.3. Human experiments

Rationale

A few sparse and sometimes contradictory reports exist describing the effect of prostacyclin treatment to increase the endogenous fibrinolytic potential in vivo. Experimentally, in dogs (Utsumomiya, Krausz, Valeri et al. 1989) and rats (Hussaini and Moore 1985) prostacyclin infusion was reported to increase the plasma fibrinolytic activity as measured by the euglobulin clot lysis assay (ECLT). Similar effects have been observed with certain prostacyclin analogues including iloprost in rats (Witt and Baldus 1989) and taprostene in rats (Schneider 1987). Positive reports of a profibrinolytic effect of prostacyclin infusion in patients with peripheral arterial disease (PAD) originated predominantly from two collaborating groups: repeated prostacyclin infusions decreased the ECLT by 50 % after 3 hours (Dembinska-Kiec, Kostka-Trabka and Gryglewski 1982). In PAD patients receiving 3 hour long infusions of prostacyclin a similar effect was reported, accompanied by a decrease in plasma plasminogen and antiplasmin levels. This served as evidence for enhanced thrombolytic activity although fibrinogen levels were unaffected (Szczeklik, Kopec, Sladek et al. 1983). In more recent experiments in PAD patients prostacyclin and iloprost infusions caused modest decreases in euglobulin clot lysis times (17 % and 19 % decrease respectively) compared to controls (12 % decrease) (Musial, Wilcyznska, Sladek et al. 1986). Other groups have been unable to show an effect of iloprost infusion on the ECLT in healthy volunteers (Belch, Greer, McLaren et al. 1984), and clau-

dication patients (Hay, Waller, Carter et al. 1987). When comparing these reports 3 main variables might explain the different results, apart from the test subjects:

1)

Not all work takes into account the normal diurnal variation in endogenous fibrinolytic activity (60 % decrease in ECLT between 8.00 a.m. and 8.00 p.m.) (Grimaudo, Hauert, Bachman et al. 1988), still a source of false positive results (Grimaudo, Omri, Kruithof et al. 1988)

2)

The lack of standardization of the ECLT assay (discussed later) and

3)

The ECLT assay time point in relation to the start of the infusion.

The purpose of this series of experiments was to closely examine the effect of taprostene infusions, at two dose levels, on the endogenous fibrinolytic activity in healthy male volunteers under placebo controlled, blinded conditions by frequent ECLT sampling. Moderate stimulation of fibrinolysis might be expected to cause a decrease in plasma antiplasmin activity, and marked stimulation a decrease in fibrinogen levels. For this reason the two factors were assayed here. In addition the clot lysis time is, in part, dependent on the density of the fibrin net and hence the concentration of fibrinogen in the blood samples, another justification for this assay to quantify a variable with potential to affect the main assay, the ECLT.

Critique ECLT assay

The euglobulin clot lysis assay and the fibrin plate assay are by far the most ubiquitous tests of the "endogenous fibrinolytic activity". Both assays require the removal of plasmatic inhibitors from plasma, by euglobulin fraction formation in order to unveil fibrinolytic activity. The chosen methodology of euglobulin fraction precipitation differs between laboratories, e.g. pH of precipitation between 5.2 and 6.0, and not surprisingly, the composition of the euglobulin fraction, produced from a complex starting material like plasma, is poorly characterized with regard to the degree of separation of individual factors involved in fibrinolytics, e.g. PAI and C_1-inactivator (Kluft 1979; Kluft and Jie 1986). Parenthetically but interestingly although scu-PA is normally present in human plasma, it seems to play only a minor role in the lysis of euglobulin clots (Grimaudo, Hauert, Bachman et al. 1988) although this may be underestimated however as thrombin, added PAI to form the starting clots, inactivates r-scu-PA (Pannell and Gurewich 1986) especially under the ideal ECLT assay conditions (37°C, many hours incubation). However as u-PA antigen levels do not undergo diurnal variation in contrast to t-PA and PAI-1 antigen levels, the observed diurnal variation in endogenous fibrinolytic rate is attributed primarily to the net balance of activity of the latter two (Andreotti, Davies, Hackett et al. 1988). Furthermore as PAI, which forms a 1:1 stoichiometric inhibitor complex with t-PA, is usually present in excess in plasma of sedentary healthy individual small changes in t-PA or PAI activity will go unnoticed. On stimulation of t-PA release, by venous occlusion or exercise a rapid increase in t-PA surmounts PAI inhibition and minor increases

in t-PA activity should be detected by the ECLT by the assay.

It can be stated categorically that on the basis of the results of the clottable fibrinogen (Clauss) assay and the AP activity assay that there is no evidence of extensive activation of the fibrinolytic system by taprostene here, however as the physiological fibrinolytisis is fibrin specific, stimulation would not cause degradation of clotting factors as seen after treatment with streptokinase. The ECLT results suggest that moderate stimulation of the endogenous fibrinolysis system occurred after high dose taprostene infusion. This may be of clinical relevance as synergism between exogenous fibrinolytic drugs (tPA and scuPA) has been reported (Collen 1988 b).

5 Summary and Conclusion

The results of these studies, under the limitations imposed by the short observation periods (hours), species difference and perfect health of the experimental animals used, showed that a number of effects are exerted by taprostene in vivo which render it a useful adjuvant in thrombolytic therapy with r-scuPA. Taprostene cotreatment reduced infarct size and arrhythmias after r-scuPA recanalization of canine coronary arteries, without markedly affecting its thrombolytic specificity, selectivity or intensity. Treatment with taprostene greatly potentiated the antiocclusive effect in a coronary artery thrombosis model when given with r-scuPA, and in healthy volunteers taprostene infusion moderately stimulated endogenous fibrinolysis possibly by stimulating endogenous tPA release, which has been shown elsewhere to act synergistically with scuPA in thrombolysis, enabling the doses to be reduced. The potential disadvantages of cotreatment of taprostene with r-scuPA are probably mostly related to the dose of the former, and its hypotensive effect, especially in acute myocardial infarction where cardiogenic shock is not uncommon.

The relatively short chemical half lifes of the 2 compounds in plasma could be used to advantage to terminate their actions when required, although an unexpected prolongation of the biological effects of combined therapy cannot be excluded on the basis of results from these experiments.

The current search for modified ("third generation") thrombolytic agents with enhanced fibrin binding-affinity should yield compounds with greater potency and thrombolytic success rates, assuming that

thrombosis is the occlusive mechanism in greater than 90% of AMI patients. These agents will probably be burdened by an increased rate of cerebral and other spontaneous bleeding events as the selective effect of a "short sharp" local thrombolytic effect on recent thrombi is lost and old haemostatic plugs are increasingly affected. Exclusion criteria for therapy could be restricted to reduce this risk.

The ubiquity of responses, desirable and otherwise, in vivo to systemic prostacyclin therapy will remain a major drawback. The side effects must be weighed against the expected beneficial effect for each disease state, and the balance might be achieved in the dosing in AMI.

In the treatment of AMI rapid recanalization of the infarct related artery remains the primary acute goal and maintenance of patency is a long term goal. Thrombin inhibitors should prove to be valuable adjuvants in thrombolytic therapy due to thrombin's central role in both fibrin and platelet haemostasis. Antineutrophil and antiinflammatory drugs may reduce the reperfusion injury attributed in part to PMN but the effects of long term treatment on infarct resolution, scar-tissue formation and normal immune defense remains to be elucidated. In hospital mortality after AMI has been reduced dramatically to approximately 6 to 10% at present, to which thrombolytic therapy has made a major contribution. The potential benefit of adjuvant therapy in thrombolysis is therefore relatively small as a percentage but great in terms of numbers of lives saved.

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Deep gratitude is due to those who showed patience, guidance and the gift of being able to provoke thought in others:

Dr. Brian Woodward, Prof. L. Flohé, Prof. H. Giertz, Prof. R. Flower and Dr. J. Schneider.

To those who shared the burden and in doing so made the last few years a pleasure:

Andrea Plum, Ulla Fincke, Hans Stahlberg, Karin Lützeler and Simone Claßen.

To those not involved but who unselfishly shared their insight:

Prof. van de Werf, Leuven;
Dr. B. Lucchesi, Michigan;
Prof. Collen, Leuven;
Dr. D. Yellon, London;
Prof. Bachman, Lausanne;
Dr. Kruithof, Lausanne;
Dr. Friderichs, Aachen;
Dr. Günzler, Aachen;
Dr. Loschen, Aachen.

And to my family. Those that are, were and will be.

"So eine Arbeit wird eigentlich nie fertig, man muß sie für fertig erklären, wenn man nach Zeit und Umständen das Möglichste getan hat."

Johann Wolfgang Goethe
Italienische Reise